VOLTAGE-DEPENDENT K^+ CURRENT IN RAT RENAL RESISTANCE ARTERIOLES: ALTERATIONS IN HYPERTENSION AND PREVENTION WITH GENE THERAPY

By

JEFFREY R. MARTENS

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This dissertation is dedicated to my wife and parents. To my parents, your love has provided me with the confidence to pursue my goals. To my wife, Lori, your love and support keeps me going. It is your presence in my life that gives me perspective on that which is important during our time on this earth.

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VOLTAGE-DEPENDENT K^+ CURRENT IN RAT RENAL RESISTANCE ARTERIOLES: ALTERATIONS IN HYPERTENSION AND PREVENTION WITH GENE THERAPY

By

Jeffrey R. Martens

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Chairman: Craig H. Gelband, Ph.D.
Major Department: Pharmacology and Therapeutics

Essential hypertension is characterized by a near normal cardiac output but an increase in total peripheral resistance. In turn, total peripheral resistance is controlled directly by the diameter of the small arteries and arterioles like those in the kidney. The dynamic regulation of renal vessel diameter is governed by the contractile state of the vascular smooth muscle cells which line the artery walls. Voltage-dependent K^+ (Kv) channels have been demonstrated to regulate smooth muscle membrane potential and thereby control smooth muscle tone. However, little data are available on Kv channel function in the renal vasculature and their potential role in essential hypertension. The objectives of this study are (1) to biophysically, pharmacologically, and biochemically identify and characterize Kv conductances in vascular smooth muscle cells from rat renal resistance arterioles, (2) to detail potential changes in Kv current and membrane potential

in genetic and nongenetic models of hypertension, and (3) to determine the effects of antihypertensive gene therapy on Kv current changes and renovascular alterations in essential hypertension.

Electrophysiological experiments revealed the presence of two overlapping components of Kv current. A transient component which activated at depolarized potentials appeared superimposed on a non-inactivating delayed rectifier-like component. The steady-state activation and inactivation, rate of recovery from inactivation, sensitivity to 4-amionpyridine, and sensitivity to α-dendrotoxin and mast cell degranulating peptide were similar for both components of Kv current. Western blot analysis demonstrated the presence of Kv1.2 and Kv1.4 α-subunits in rat renal resistance vessels and co-immunoprecipitation experiments confirmed the heteromultimeric association of these proteins *in vivo*.

Comparison of K^+ currents and electrical properties between normotensive and hypertensive rat models demonstrated that (1) smooth muscle cells from spontaneously hypertensive rats (SHRs) and deoxycoritcosterone acetate (DOCA) treated rats were ~ 20 mV more depolarized than control, and (2) the calcium activated K^+ current (K_{Ca}) was increased and the Kv current was decreased in hypertensive models compared to normotensive controls. The change in Kv current as well as the onset of other renovascular and cardiac pathophysiological changes were prevented by treating pre-hypertensive SHRs with angiotensin II type 1 receptor antisense.

CHAPTER 1
INTRODUCTION

The diagnostic measure of hypertension is an elevation in blood pressure which is determined by cardiac output and total peripheral resistance (TPR). Essential hypertension is characterized by a near normal cardiac output but an increase in total peripheral resistance (Frohlich, 1973). TPR in turn is controlled directly by vessel diameter particularly that of the small arteries and arterioles. The dynamic regulation of vessel diameter is governed by the contractile state of the vascular smooth muscle cells which line the vessel walls. Smooth muscle contraction, like that of striated muscle, is controlled by increases in intracellular Ca^{2+} concentration. Calcium binds to calmodulin at four distinct "EF-hand" sites, inducing a conformational change in the binding protein (Mani & Kay, 1996). The Ca^{2+}-calmodulin complex is then able to activate myosin light chain kinase which transfers a phosphate from ATP to a serine at position 19 on the 20-kD light chain of myosin (Somlyo & Somlyo, 1994). This allows the contractile myofilaments (actin and myosin) to interact and the initiate crossbridge cycling. As long as intracellular ATP is present and the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) remains elevated over basal levels, contraction of the smooth muscle is maintained.

In vivo, vascular smooth muscle exists in a state of partial contraction. In this state, termed myogenic tone, levels of $[Ca^{2+}]_i$ exceed that observed in the relaxed state (Bolton, 1979; Marin, 1993). These levels of $[Ca^{2+}]_i$ are maintained by the balance

between calcium influx and extrusion (Khalil *et al.*, 1989; Orallo, 1996; Karaki *et al.*, 1997)). A stimulatory rise in $[Ca^{2+}]_i$ may lead to further contraction of the vascular smooth muscle. This process is termed excitation-contraction coupling of which there are two recognized mechanisms. The first, electromechanical coupling, operates through changes in the vascular smooth muscle cell's membrane potential. The second, pharmacomechanical coupling, operates through agonist stimulation of second messenger signaling pathways to increase $[Ca^{2+}]_i$ and does not necessarily involve prior membrane depolarization. These mechanisms are not independent of each other (Somlyo & Somlyo, 1994); however, both require an increase in myoplasmic free Ca^{2+}. The two pathways leading to this increase in $[Ca^{2+}]_i$ are (1) release from intracellular stores, and (2) influx from the extracellular space. The release of Ca^{2+} from the sarcoplasmic reticulum is associated with a transient contraction. Whereas, maintained contraction is dependent on Ca^{2+} influx across the sarcolemma. Although, it has been demonstrated that agonist stimulated $[Ca^{2+}]_i$ levels and contraction are altered in hypertension, it is the maintained contraction that is important in the long term regulation of arterial pressure.

Two major routes exist for the stimulated entry of extracellular Ca^{2+} in vascular smooth muscle; voltage gated Ca^{2+} channels and receptor operated channels (ROCs). Other mechanisms of Ca^{2+} entry have been identified (i.e. non-selective cation channels, calcium release activated calcium channels, and stretch activated channels). However, the nature of their role in the control of vascular tone is not clearly established and it has yet to be determined if these mechanisms are truly separate and unique pathways of Ca^{2+} entry. For example, it has been proposed that Ca^{2+} entry through ROCs may not be distinct from that of voltage-dependent Ca^{2+} channels or that the main function of ROC's

is to shift the membrane potential into the range where voltage-dependent Ca^{2+} channels have an increased conductance (Bolton *et al.*, 1988; Nelson *et al.*, 1990b; Karaki *et al.*, 1997). Nevertheless, Ca^{2+} influx through voltage-dependent Ca^{2+} channels has been established as important in the control of vascular tone.

Since Ca^{2+} channels in vascular smooth muscle are strongly voltage-dependent, their function is closely linked to changes in membrane potential. Plasmalemma membrane potential is determined by the permeability of several ions. While Na^+ flux is controlled mainly by pumps and exchangers (Na^+-Ca^{2+} exchanger or Na^+/K^+ ATPase), a variety of ion channels permit the passage of Ca^{2+}, K^+ and Cl^- across the membrane. This review will focus on the nature of these channels in vascular smooth muscle and how changes in their current may contribute to membrane potential alterations in hypertension.

Voltage-Dependent Ca^{2+} Channels

To date, two subtypes of voltage-dependent Ca^{2+} channels have been identified in vascular smooth muscle: L- and T-type (Orallo, 1996). L-type Ca^{2+} channels in vascular smooth muscle are activated by relatively strong depolarizations, inactivate slowly and incompletely, and are blocked by dihydropyridines. In contrast, T-type Ca^{2+} channels are activated at more negative potentials, exhibit rapid, voltage-dependent inactivation, and are insensitive to dihydropyridines. L-type Ca^{2+} channels appear to be the major Ca^{2+} influx pathway in vascular smooth muscle. Entry through this channel is important in the control of resting tone (Rubart *et al.*, 1996). The influx through T-type Ca^{2+} channels is small, transient, and likely to be inactivated at rest. Therefore, the remaining discussion will focus on L-type Ca^{2+} channels.

In smooth muscle, L-type Ca^{2+} channels are composed of multiple subunits: α_1, α_2/δ, β (Hofmann & Klugbauer, 1996). The α_1 subunit is the pore forming central component, necessary for ion selectivity, voltage sensing, activation and inactivation (Catterall, 1988). The α_1 also contains binding sites for the organic (dihydropyridines, pheylalkylamines, and benzothiazepines) and inorganic (Cd^{2+}, Ni^{2+}) Ca^{2+} channel blockers (Bosse *et al.*, 1992). The highly conserved α_2/δ subunit is a dimer that is linked together with a disulfide bond and may be involved in membrane anchoring. The β subunit appears to be a modulatory subunit that shifts the voltage dependence of activation and inactivation to more negative membrane potentials (Neely *et al.*, 1993).

The dependence of the L-type Ca^{2+} channels on membrane potential is important in the control of vascular tone (Nelson *et al.*, 1990b). The overlapping nature of the L-type Ca^{2+} channel activation and inactivation curves predicts a small sustained Ca^{2+} influx though this channel. This has been termed the Ca^{2+} window current and its reported values fall within the range of *in vivo* measured smooth muscle membrane potential. Therefore, it may account for the steady-state levels of $[Ca^{2+}]_i$ necessary for maintained vascular tone (Smirnov & Aaronson, 1992b; Fleischmann *et al.*, 1994). L-type Ca^{2+} channel open probability (P_o) increases sharply with membrane depolarization, changing approximately e-fold for 6-8 mV (Nelson *et al.*, 1990b). This steep voltage dependence allows the L-type Ca^{2+} channels to respond to small graded changes in membrane potential. Therefore, L-type Ca^{2+} channels provide a regulatory mechanism for the interdependence of Ca^{2+} influx, membrane potential, and vessel diameter (Khalil *et al.*, 1987; Brayden & Wellman, 1989; Nelson *et al.*, 1990b).

Smooth Muscle K^+ Channels

While depolarization of vascular smooth muscle relies on the activation of Ca^{2+} conductances, membrane repolarization is dependent on the opening of plasmalemmal K^+ channels. The opening of K^+ channels causes plasma membrane hyperpolarization and inhibition of voltage-dependent Ca^{2+} entry. Given the negative equilibrium potential for K^+, vascular smooth muscle K^+ channels not only dampen membrane depolarization but also play a prominent role in the maintenance of resting membrane potential and resulting myogenic tone. Using the patch clamp technique, at least three types of calcium-activated K^+ (K_{Ca}) channels, two types of voltage activated, calcium insensitive potassium (K_v) channels, and an ATP-dependent potassium (K_{ATP}) channel have been linked to repolarization in vascular smooth muscle. In addition, an inwardly rectifying K^+ channel (K_{ir}) may play a role in regulation of membrane potential (Tomita, 1988; Nelson & Quayle, 1995; Brayden, 1996;).

Ca^{2+}-Activated K^+ Channels

Several types of K_{Ca} channels that differ in unitary conductance (small, intermediate, or large conductance) and pharmacology have been identified in various non-vascular tissues. In vascular smooth muscle, the current most often described is a large conductance (≥200pS), Ca^{2+}, time, and voltage-dependent K^+ current. It has been identified in a number of vascular beds including: rabbit pulmonary artery (Okabe *et al.*, 1987), portal vein (Hume & Leblanc, 1989), ear artery (Benham & Bolton, 1986), canine coronary (Wilde & Lee, 1989), renal artery (Gelband & Hume, 1992), rat renal resistance vessels (Chapter 2, Figs. 2A,B,C), and cultured aortic cells (Toro & Stefani, 1987).

Membrane depolarization (~ 2.7 fold increase per 12-14 mV depolarization) as well as physiologically relevant increases in $[Ca^{2+}]_i$ increase the P_o of K_{Ca} channels (Nelson, 1997). Pharmacologically, external tetraethylammonium (TEA), charybdotoxin (ChTx), iberiotoxin (IbTx), EGTA, and Ni^{2+} inhibit channel activity. The molecular components responsible for the large conductance K_{Ca} current have been described. The initial cloning of this class of K_{Ca} channels was from the fruit fly *Drosophila*, and the gene has been termed slowpoke (d*slo*) (Atkinson *et al.*, 1991). Vogalis *et al.* (1996) recently cloned and expressed cDNAs encoding the α- and β-subunits of the d*slo* gene from canine smooth muscle.

The physiological role of large conductance K_{Ca} channels in vascular smooth muscle is well understood (Nelson & Quayle, 1995; Nelson, 1997). The membrane depolarization and elevation of $[Ca^{2+}]_i$ associated with vasoconstriction will cause activation of K_{Ca} channels. This activation is under partial regulatory control by small, concentrated bursts of Ca^{2+} release (Ca^{2+} sparks) from the sarcoplasmic reticulum (Nelson *et al.*, 1995). The resulting activation of these channels causes membrane hyperpolarization and relaxation of arterial tone (Brayden & Nelson, 1992). Finally, K_{Ca} channels are regulated by a number of endogenous vasoactive agents. Vasoconstrictors such as angiotensin II, thromboxane A_2, and muscarinic agonists have been linked to inhibition of K_{Ca} channels (Toro *et al.*, 1990; Kume & Kotlikoff, 1991; Scornik & Toro, 1992). Where as activators of protein kinase A (PKA) and G (PKG) appear to increase channel activity (Brayden & Nelson, 1992). This further suggests a physiological role for K_{Ca} channels in the regulation of vascular tone.

Recently, a second type of K_{Ca} channel was found to coexist with the large conductance K_{Ca} channel in vascular smooth muscle from rat renal arterioles (Gebremedhin *et al.*, 1996). This channel had a unitary conductance of 68 pS and was selectively blocked by apamin (50 nmol/L). An apamin-sensitive K^+ current has also been identified in rabbit mesenteric arteries (Murphy & Brayden, 1995). However, the role of this K_{Ca} channel needs to be elucidated.

Voltage-Dependent K^+ Channels

Kv channels comprise the largest and most diverse class of ion channels. This class can be subdivided into 3 major families based on their response to membrane voltage, pharmacology, and molecular identity. These include (1) the delayed rectifier K^+ currents, (2) the A-type K^+ currents, and (3) the inward rectifier K^+ (K_{ir}) currents, all of which have been identified and characterized in vascular smooth muscle. As the name implies, the K_{ir} channels posses unique rectification properties which allow it to play a separate function role in vascular smooth muscle. Therefore for the purpose of this discussion, the K_{ir} channels will be discussed separately.

Kv channels appear to be ubiquitously expressed. They have been identified and the current characterized in a variety of vascular beds including; portal vein (Beech & Bolton, 1989; Hume & Leblanc, 1989), coronary artery (Volk & Shibata, 1993), cerebral artery (Bonnet *et al.*, 1991), renal vasculature (Gelband & Hume, 1992; see Chapter 2), pulmonary artery (Okabe *et al.*, 1987), and the mesenteric vasculature (Smirnov & Aaronson, 1992a). The outwardly rectifiying current carried by delayed rectifier K^+ channels turns on with a brief delay following membrane depolarization to potentials positive to ~ -40 mV. This current exhibits slow C-type inactivation which involves a

conformational change in the channel pore (Jan & Jan, 1992). In contrast, the A-type K^+ current activates steeply with voltage between -90 and -60 mV and then decays spontaneously and rapidly. Inactivation of the A-type, sometimes referred to as the transient K^+, current has been termed N-type because it involves a pore blocking particle tethered to the N-terminus of the channel (Hoshi *et al.*, 1990). Both currents are insensitive to removal of extracellular Ca^{2+} and are selectively inhibited by low concentrations of 4-aminopyridine (4-AP).

The issue of multiple Kv channel subtypes within vascular smooth muscle is complex and may not be easily classified into the above mentioned subfamilies. Biophysical and pharmacological evidence suggests the existence of multiple components of Kv current within a single tissue. Also the existence of different K^+ channel distributions among various vascular beds or changes along an arterial tree have also been shown (Albarwani *et al.*, 1995; Evans *et al.*, 1996). On a molecular level, K_v channels are traditionally represented in four gene families (Kv1-Kv4) based on homology to those initially cloned from the *Drosophila* (Shaker, Shab, Shaw, and Shal genes). However, new families of gene products are rapidly being identified. Within each family alternative splicing and gene duplication give rise to many channel subtypes (Jan & Jan, 1992). In addition, the possibility of heterotetramer formation in smooth muscle may also increase diversity and further complicate the identification of such components (Russell *et al.*, 1994). To our knowledge only Kv1.2 (Hart *et al.*, 1993), Kv1.5 (Overturf *et al.*, 1994), Kv2.2 and Kv1.1 (Horowitz, B., personal communication) have been cloned and characterized in smooth muscle. However in vascular smooth muscle, the molecular

component for only Kv1.5 and Kv2.2 have been demonstrated using western blot analysis.

Voltage-dependent K^+ channels have several physiological roles in vascular smooth muscle. One important role is to dampen excitation by providing a mechanism of membrane hyperpolarization. Nelson *et al.* (1990b) calculated that due to the high input resistance of vascular smooth muscle cells, only a small number of Kv channels need to be open to limit membrane depolarization and facilitate vasorelaxation. A second important role is the contribution of Kv channels to vascular smooth muscle resting membrane potential. The steep relationship between steady state P_o and membrane voltage, as determined from the mean activation and inactivation curves, suggest the presence of a significant sustainable Kv current at rest (Nelson & Quayle, 1995). In addition, pharmacological blockade of Kv channels leads to membrane depolarization in current clamped isolated vascular smooth muscle cells as well as constriction of intact vessels (Yuan, 1995; Knot & Nelson, 1995; Gelband and Hume, 1995; Post *et al.*, 1995; Iyer *et al.*, 1996). This provides additional evidence for the role of Kv channels in the regulation of vascular smooth muscle membrane potential. Finally, it has been proposed that Kv channels are regulated by a number of endogenous vasoactive agents. Vasoconstrictors such as angiotensin II, histamine and hypoxia have been associated with inhibition of Kv channels (Ishikawa *et al.*, 1993; Gelband & Hume, 1995; Post *et al.*, 1995).

Inward Rectifier K^+ Channels

K_{ir} current has been identified in several vascular beds including cerebral (Quayle *et al.*, 1993), coronary (Robertson *et al.*, 1996), and mesenteric vasculature (Edwards &

Hirst, 1988a). Interestingly, these channels appear to be localized in small resistance size vessels (i.e. < 200 μm). The channels display a time-independent rectification that is opposite to the driving force established by the K^+ ion gradient. They open with steep voltage dependence upon hyperpolarization, however pass a small sustained outward current at potentials positive to the K^+ equilibrium potential (E_K). The channel is also very sensitive to changes in extracellular K^+ ($[K^+]_o$) possessing the ability to shift its voltage dependence of gating with changes in E_K. Micromolar concentrations of extracellular Ba^{2+} cause a voltage dependent block of K_{ir}. Inhibitors of other K^+ channels (i.e. 4-AP, TEA, glibenclimide) appear to have no effect on K_{ir}. However, millimollar concentrations of Cs^+ also appear to block channel activity.

A K_{ir} channel is yet to be cloned from smooth muscle. However, it is expected to share a similar structure to that of cloned K_{ir} channels from other tissues. K_{ir} is believed to assemble as a tetramere with each subunit having two hydrophobic segments very similar to the S5, S6 and H5 segments which comprise the pore region of the Shaker-like K^+ channels.

Similar to the molecular identity, the physiological role of K_{ir} in vascular smooth muscle is not well defined. As mentioned above, K_{ir} channels do conduct a small amount of outward current at potentials positive to E_K. Therefore, in the absence of depolarizing factors they may contribute to the maintenance of resting membrane potential. Low concentrations of Ba^{2+}, selective for K_{ir} have been shown to depolarize vascular smooth muscle from small cerebral and coronary arteries (Hirst *et al.*, 1986; Hirst & Edwards, 1989; Brayden, 1996). However, this is in the absence of transmural pressure which may

be significant (Brayden, 1990; McCarron & Halpern, 1990). The unique sensitivity of K_{ir} to changes in extracellular K^+ may suggest another physiological role for this channel. Several pathophysiological conditions such as hypoxia or ischemia result in the elevation of extracellular K^+ ($[K^+]_o$). K_{ir} is one mechanism of vasorelaxation by which vascular smooth muscle cells may respond to this increase (Chen *et al.*, 1972; Edwards *et al.*, 1988b; McCarron & Halpern, 1990). However, a second mechanism, activation of the Na^+/K^+ pump, may underlie the vasodilatory response. Nevertheless, the physiological role and molecular identity of K_{ir} in vascular smooth muscle remains unclear.

ATP-Sensitive K^+ Channels

A potassium current that is voltage independent and inhibited by the noncovalent binding of ATP has been identified in several vascular beds including the pulmonary (Clapp & Gurney, 1992), coronary (Xu & Lee, 1994; Dart & Standen, 1995), and mesenteric vasculature (Silberberg & van Breemen, 1992; Quayle *et al.*, 1994). While all of the currents from these tissues are blocked by glibenclimide, the nature of the single channel conductance which underlies K_{ATP} is unclear. The literature describes two populations of channels of varying unitary conductance (large; 130-260 pS : small; 10-30 pS) depending on the preparation, vascular bed, recording method, and Ca^{2+} sensitivity (Zhang & Bolton, 1996). K_{ATP} channels are composed of at least two subunits which are believed to co-assemble as heterotetramers. Two subunits similar to the truncated Shaker-like K_{ir} proteins combine with the sulfonylurea receptor to form functional K_{ATP} channels (Inagaki *et al.*, 1995). These subunits have recently been cloned from smooth muscle (Horowitz, B.; personal communication) and their heterologous expression will certainly provide further insight into their unitary conductance and cellular regulation.

Although ATP inhibits channel activity, adenosine diphosphate (ADP) increases K_{ATP} current, at least in the portal vein (Kajioka *et al.*, 1991; Pfrunder *et al.*, 1993; Kamouchi & Kitamura, 1994). In addition adenosine has been shown to activate K_{ATP} and cause vasodilation (Dart & Standen, 1995). Also, activation of K_{ATP}, via a cAMP/PKA second messenger pathway contributes to the calcitonin gene-related (CGRP) mediated relaxation of rabbit mesenteric arteries (Nelson *et al.*, 1990a; Quayle *et al.*, 1996). Endothelial factors such as prostacyclin and nitric oxide (NO) also appear to activate K_{ATP} channels (Murphy & Brayden, 1995). The NO dependent activation of K_{ATP}, like that of CGRP appears to be transduced via a second messenger pathway, but in this case involving cGMP (Miyoshi & Nakaya, 1994; Kubo *et al.*, 1994). In contrast to the agents that cause arterial relaxation, a number of endogenous vasoconstricting agents including Ang II (Miyoshi & Nakaya, 1991), endothelin (Byrne & Large, 1987), and vasopressin (Wakatsuki *et al.*, 1992) have been shown to inhibit K_{ATP}. However, the mechanism of this inhibition is yet to be elucidated.

The functional role of K_{ATP} in vascular smooth muscle is diverse. It has been suggested that K_{ATP} plays an important role in reactive hyperemia, endotoxic shock, and in the metabolic regulation of blood flow. Taking into account the large number of endogenous vasoactive agents that target K_{ATP}, this channel must be important in the regulation of vascular tone. However, this appears to be dependent on the vascular bed. For example, glibenclimide constricts arteries from the coronary and mesenteric vasculature but does not affect those from the renal, cerebral, or pulmonary vascular beds (Nelson & Quayle, 1995). In addition, a number of K^+ channel opening drugs appear to

cause activation of K_{ATP}. These drugs including minoxidile, dizoxide, pinacidil, and cromakalim cause direct vasodilation and have been used as antihypertensive agents.

Smooth Muscle Cl^- Channels

Two major types of chloride selective conductances have been identified in vascular smooth muscle. One is a voltage-dependent chloride current that has been reported in vascular smooth muscle cells (Soejima & Kokubun, 1988; Yamazaki *et al.*, 1998). The chloride conductance most often reported in vascular smooth muscle is that of a Ca^{2+} dependent Cl^- ($I_{Cl(Ca)}$) current with a small unitary conductance of ~ 2-3 pS (Large & Wang, 1996). Patch clamp recordings first identified $I_{Cl(Ca)}$ as one component of current underlying the norepinephrine evoked depolarization of rat anaccoxycygeus muscle cells (Byrne & Large, 1987). Since then $I_{Cl(Ca)}$ has been identified in a number of vascular tissues including; portal vein (Byrne & Large, 1988; Pacaud *et al.*, 1989a; Leblanc & Leung, 1995), ear artery (Amedee *et al.*, 1990a), mesenteric artery (Klockner & Isenberg, 1991), pulmonary artery (Leblanc & Leung, 1995), and rat renal resistance arteries (Gordienko *et al.*, 1994).

$I_{Cl(Ca)}$ is strongly Ca^{2+} dependent with this relationship varying between tissue and species. One study in rat portal vein reports the half-maximal activation of $I_{Cl(Ca)}$ at ~ 365 nmol/L (Pacaud *et al.*, 1992). However, it appears that $I_{Cl(Ca)}$ is not as sensitive to $[Ca^{2+}]_i$ as K_{Ca} (Large & Wang, 1996). Also, it has been suggested that unlike K_{Ca}, activation of $I_{Cl(Ca)}$ may require both Ca^{2+} and a second unknown substance. This claim is based on the rapid rundown of $I_{Cl(Ca)}$ in whole cell and excised patch recordings. This decrease in channel activity was prevented using nystatin perforated patches (Amedee *et al.*, 1990b). In addition $I_{Cl(Ca)}$ is weakly voltage dependent with an e-fold increase in P_o for an ~ 100

mV change in membrane potential (compare this to Ca^{2+} and Kv current) (Large & Wang, 1996).

In order to understand the functional role of $I_{Cl(Ca)}$ it is necessary to examine the Cl^- ion distribution in vascular smooth muscle cells. The presence of secondary active Cl^- transport mechanisms (i.e. Cl^-/HCO_3^- exchanger and the Na^+-K^+-$2Cl^-$ cotransporter) within the plasmalemma of smooth muscle cells results in the accumulation of intracellular chloride above that expected from passive distribution (Casteels, 1971; Jones, 1980; Koncz & Daugirdas, 1994). Therefore, the Cl^- equilibrium potential is ~ -20 mV. Since the resting membrane potential of vascular smooth muscle cells is more negative than this (~ -50 mV), activation of Cl^- channels will lead to a net Cl^- efflux resulting in membrane depolarization. $I_{Cl(Ca)}$ is activated in vascular smooth muscle by a number of vasoactive agents including; norepinephrine (Byrne & Large, 1987; Byrne & Large, 1988), ATP (Pacaud & Loirand, 1995; Wang *et al.*, 1997), endothelin (Klockner & Isenberg, 1991; Gordienko *et al.*, 1994), histamine (Wang & Large, 1993), and vasopressin (Van Renterghem & Lazdunski, 1993). This activation appears to be mediated by the stimulatory release of Ca^{2+} from intracellular stores (Large & Wang, 1996). The resulting membrane depolarization may recruit more voltage dependent calcium channels to open producing contraction. It has also been proposed that $I_{Cl(Ca)}$ may be activated by Ca^{2+} sparks from the SR. This current termed spontaneous transient inward currents (STICS) has been recorded in several vascular tissues (Wang *et al.*, 1992; Hogg *et al.*, 1993). Finally, $I_{Cl(Ca)}$ may play a role in the regulation of resting membrane potential. It is hypothesized that membrane permeability to Cl^- may explain why the resting membrane potential of vascular smooth muscle is positive to E_K.

Recently, Nelson *et al.* (1997) has suggested that a niflumic acid (niflumic acid is a selective inhibitor of $I_{Cl(Ca)}$) insensitive Cl^- current may contribute to myogenic tone in rat cerebral arteries. However much work is needed to identify specific Cl^- channels contributing to vascular tone. Nonetheless, the most probable role for $I_{Cl(Ca)}$ is its contribution to excitatory agonist induced vasoconstriction (Large & Wang, 1996; Yuan, 1997).

Ion Channel Alterations in Hypertension

As mentioned previously, essential hypertension involves a gradual and sustained increase in total peripheral resistance. These changes in vessel diameter must involve alterations in the contractile state of vascular smooth muscle. In both human and animal models of essential hypertension it has been demonstrated that the resting vascular tone is elevate and the contractile response to a number of physiological stimuli is enhance compared to normotensive controls. Both of these changes will contribute to the increased blood flow resistance. A number of cellular mechanisms in vascular smooth muscle may contribute to these changes in hypertension (Fig. 1-1). The focus of this discussion will be alterations in plasma membrane ion channels.

To date, the majority of fluorescence and electrophysiological studies of vascular changes in essential hypertension have been performed on large conduit vessels. Conflicting results regarding changes in contractility (Bohr, 1974), resting membrane potential (Hermsmeyer, 1976; Steikel, 1989; Lamb & Webb, 1989; Rusch *et al.*, 1992; See Chapter 3), basal $[Ca^{2+}]_i$ (Sugiyama *et al.*, 1986; England *et al.*, 1993; See Chapter 3), and the onset of ion channel alterations (Ohya *et al.*, 1996) in conduit vessels from models of essential hypertension may be due to differences in the vascular bed studied.

Mulvaney *et al.* (1990) has suggested that the increased contractility observed in vascular beds from hypertensive models must reside in the resistance vessels. Moreover, the large conduit vessels play a less significant role in setting peripheral vascular resistance than do smaller resistance vessels (Mulvany & Aalkjaer, 1990b). Studies of clinically relevant resistance vessels (i.e. renal or cerebral vasculature) may provide further insight into the role of ion channel alterations in the development and maintenance of essential hypertension.

In the literature, a multitude of evidence suggests that altered ionic permeabilities exist in vascular smooth muscle in hypertension (Jones, 1973; Frohlich, 1973). Using the patch clamp technique and vascular smooth muscle cells dissociated from conduit arteries , it has been demonstrated that there is an increased L-type Ca^{2+} current density in vascular smooth muscle cells from the spontaneously hypertensive rat (SHR), a model of human essential hypertension (Rusch & Hermsmeyer, 1988; Ohya *et al.*, 1993; Wilde *et al.*, 1994; Cox & Lozinskaya, 1995; Lozinskaya & Cox, 1997). In addition, it has also been shown that the K_{Ca} current density in aorta from hypertensive rats is larger than control (Rusch *et al.*, 1992). This alteration was observed in single-channel recordings of the K_{Ca} current from excised aortic membrane patches in which a greater Ca^{2+} sensitivity of the SHR K_{Ca} was demonstrated (England *et al.*, 1993). This increase in K_{Ca} may also be due to an upregulation of channel expression resulting from a change in some post-transcriptional event (Liu *et al.*, 1997). Finally, Ohya *et al.* (1996) have reported an impaired action of levocromakalim on K_{ATP} channels in smooth muscle cells from the SHR. This data suggests that alterations in K_{ATP} function or density may exist in hypertension.

Reversal of Ion Channel and Pathophysiological Alterations in Hypertension

Hypertension produces pathophysiological changes which are often responsible for the mortality associated with the disease. However, it is unclear if normalizing blood pressure with conventional therapy is effective in reversing the pathophysiological damage. The duration and initiation of treatment, site of administration, and agent used all appear to influence the reversal of the pathophysiological alterations associated with hypertension. A major question regarding ion channel alterations in vascular smooth muscle cells and the development of essential hypertension is one of cause vs. effect. That is do the observed changes in ionic current underlie the development of hypertension or do they simply follow changes in blood pressure. The relationship between blood pressure changes and ion channel alterations has been addressed in a few studies. Rusch and Runnells (1994) showed that normalization of high blood pressure in SHR using ramipril, an angiotensin-converting enzyme inhibitor (ACE), reversed the increase in K_{Ca} observed in the aorta. In addition, Ohya *et al.* (1996) reported that the impaired action of levcromacalim on K_{ATP} channels in SHR was corrected with pharmacological antihypertensive treatment including; hydralazine, Ca^{2+} channel blockers, and ACE inhibitors.

Rationale

Hypertension is a leading cause of death and disability among adults in the United States. Despite years of intensive investigation, in most cases the etiology of hypertension remains unknown. Essential hypertension is characterized by a near normal cardiac output but an increase in total peripheral resistance (Frohlich, 1973). The elevation in total peripheral resistance associated with hypertension may result from

alterations in vasomodulatory substances, passive or genetic modifications in blood vessel structure, or intrinsic changes in the control of vascular tone (Khalil *et al.*, 1989). The dynamic regulation of vascular tone is governed by the contractile state of the smooth muscle cells which line blood vessel walls. In turn, the excitation and contraction of vascular smooth muscle depends on cellular membrane potential which is known to be altered in the blood vessels of hypertensive animal models (Hermsmeyer, 1976). Kv channels have been shown to regulate smooth muscle membrane potential and thereby contribute to the control of vascular smooth muscle tone (Brayden *et al.*, 1991; Nelson & Quayle, 1995).

The results of earlier renal transplant studies in humans and animals suggest that in a proportion of cases with essential hypertension there exists a defect in the structure and/or function of the kidney (Dahl *et al.*, 1972; Bianchi *et al.*, 1979; Curtis *et al.*, 1983). The small arteries (< 200 μm) of the kidney which provide the greatest resistance to blood flow are an important vessel to study. Alterations in renal blood flow are known to influence fluid volume regulation, secretion of a number of important neurohumoral substances, and arteriolar resistance all of which may be involved in the maintenance of hypertension. Given this, little data are available on Kv channel function in the renal vasculature and its potential role in essential hypertension. This project has therefore three specific aims.

Specific aim #1 In the first part of this project, the biophysical, pharmacological, and biochemical characterization of Kv current in rat renal resistance arteries was carried out.

The results of this study titled *"Voltage-dependent K^+ current in rat renal resistance arteries: Evidence for heteromultimeric formation"* are presented in Chapter 2.

Specific Aim #2 In the second part of this project, we detailed changes in membrane potential and K^+ current in vascular smooth muscle cells isolated from rat renal resistance arteries of hypertensive animal models. Specifically, we studied Kv channel alterations in genetic and nongenetic models of hypertension. The results of this study titled *"Alterations in rat renal resistance artery membrane potential and K^+ channels in genetic and nongenetic hypertension"* are presented in Chapter 3.

Specific Aim #3 In the third part of this project, the effects of antihypertensive gene therapy on renovascular alterations and Kv current changes in a genetic model of essential hypertension are investigated. This study shows that interruption of the renin-angiotensin system in pre-hypertensive animals attenuates the elevation in blood pressure and prevents the development of Kv current alterations. The results of this study titled *"Prevention of renovascular and cardiac pathophysiological changes in hypertension by angiotensin II type 1 receptor antisense gene therapy"* are presented in Chapter 4.

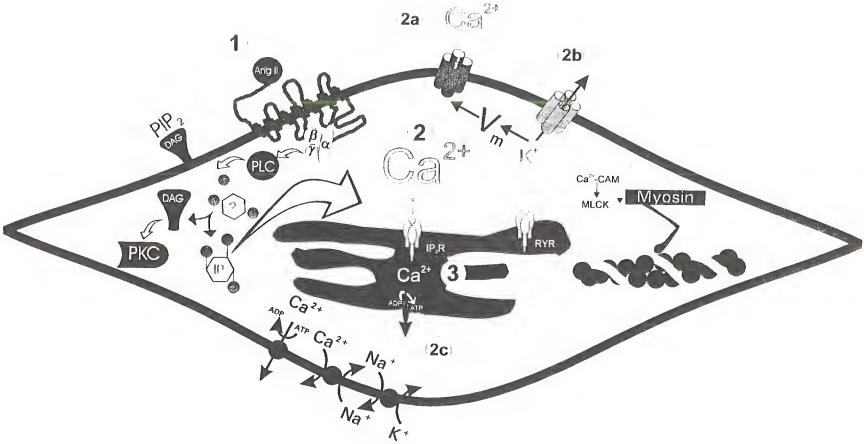

Figure 1-1. Cellular mechanisms in vascular smooth muscle cells which may contribute to contractile alterations in essential hypertension: 1) increased sensitivity to circulating hormones such as angiotensin II. 2) increased levels of myoplasmic free Ca^{2+}; this may result from 2a) increased Ca^{2+} current, 2b) decreased K^+ current, 2c) altered activity of pumps and exchangers in the SL or SR. 3) increased Ca^{2+} loading of the SR, or 4) increased Ca^{2+} sensitivity of the contractile myofilaments.

CHAPTER 2
VOLTAGE-DEPENDENT K^+ CURRENT IN VASCULAR SMOOTH MUSCLE CELLS FROM RAT RENAL RESISTANCE ARTERIOLES: EVIDENCE FOR HETEROTETRAMER FORMATION

Introduction

The presence of a voltage-dependent K^+ (Kv) current that is sensitive to 4-aminopyridine (4-AP) appears to be ubiquitous in vascular smooth muscle cells. However, the nature of this current has been shown to vary depending on the vascular bed or segmental location along an arterial tree (Nelson & Quayle, 1995; Brayden, 1996; Archer *et al.*, 1996; Patel *et al.*, 1997). In addition, biophysical and pharmacological evidence suggests the existence of multiple components of Kv current within single smooth muscle cells (Carl, 1995). Both transient and delayed rectifier Kv currents have been identified (Beech & Bolton, 1989; Lang, 1989; Clapp & Gurney, 1991; Gelband & Hume, 1992; Smirnov & Aaronson, 1992a; Gordienko *et al.*, 1994). While many studies have focused on the macroscopic current properties, very little work has been aimed at identifying Kv channel subtypes which may underlie these native currents.

Kv channels comprise the largest and most diverse class of ion channels. A large number of Kv channel genes and their splice variants have been reported (Chandy & Gutman, 1993; Roberds *et al.*, 1993). This diversity complicates the correlation of Kv channel cDNA's with endogenous currents within a vascular tissue. The process of assigning molecular counterparts to Kv current components *in vivo* is further obscured by

the potential for heteromeric formation or modulation by accessory subunits. K^+ channel stoichiometry was determined to be tetrameric with four α subunits combining to form a functional channel (MacKinnon, 1991). Recent electrophysiological evidence shows that both identical (homomeric) and non-identical (heteromeric) Kv α subunits can co-assemble (Timpe *et al.*, 1988; Christie *et al.*, 1990; Isacoff *et al.*, 1990; Ruppersberg *et al.*, 1990). The biophysical and pharmacological properties of Kv heterotetramers are often unique compared to homotetrameric channels (McCormack *et al.*, 1990). In addition, β subunits are now known to couple with Kv channels altering both membrane expression and channel kinetic properties (Rettig *et al.*, 1994; England *et al.*, 1995a; England *et al.*, 1995b; Majumder *et al.*, 1995; McCormack *et al.*, 1995; Heinemann *et al.*, 1996). To date, direct *in vivo* evidence for heteromeric association between Kv α subunits or with accessory β subunits has not been demonstrated in vascular smooth muscle. In this study we have characterized the Kv current in rat renal resistance vessels and provide the first *in vivo* evidence of heteromeric formation in vascular smooth muscle.

Materials and Methods

Smooth Muscle Cell Isolation

Male rats were killed by decapitation and the right and left kidneys were removed and placed in cold, oxygenated (95% O_2-5% CO_2) physiological saline solution (PSS). Renal resistance vessels were dissected free from the kidney, cut into small pieces (~ 4 mm), and incubated at room temperature in Ca^{2+}-free PSS for 30 minutes. The pieces were subsequently resuspended in Ca^{2+}-free PSS digestion buffer for 20-30 minutes at

37°C. The Ca^{2+}-free PSS digestion buffer contained (mg/15 ml) collagenase (151 units/mg Worthington Biochemical Corp., Freehold, NJ.) 4.5, bovine serum albumin 30, trypsin inhibitor 30, ATP (sodium salt) 1.7, and protease (type XXIV, Sigma Chemical Co., St. Louis, Mo.) 1.5. After the digestion, the pieces were gently triturated until a large number of elongated smooth muscle cells were observed. The isolated cells were collected and stored at 4°C until used. Cells were used between 2 and 10 hours after isolation.

Current Recording and Analysis

Single cells were voltage-clamped, and membrane currents were measured using the whole-cell patch-clamp technique (Hamill *et al.*, 1981). Voltage-clamp command potentials were applied to the cells and membrane current recorded using an Axopatch-1D patch-clamp amplifier (Axon Instruments, Burlingame, CA). Membrane current was digitized on-line (10.0 kHz) with in analog-to-digital interface (Labmaster TL-1 DMA interface, Axon Instruments) and filtered at 2.0 kHz. Data analysis was performed with pCLAMP 6.0 software (Axon Instruments). All experiments were performed at room temperature. Gordienko *et al.* (1994) has demonstrated the presence of both a calcium activated K^+ (K_{Ca}) current and a Kv current in rat renal resistance arterioles. The K_{Ca} current may be blocked using either tetraethylammonium (TEA) shown in Figures 2-1 and 2-2 or charybdotoxin (ChTx) as shown in Figure 2-3. For this study, isolation of the Kv current was achieved using 10 mmol/L TEA in the bath solution and replacing Ca^{2+} with equal concentrations of Mg^{2+} (see solutions).

Immunoprecipitation of Kv1.2 Channels and Associated α Subunits

Renal arterioles were hand homogenized on ice in homogenization buffer containing (in mmol/L): 250 sucrose, 50 MOPS, 0.1 PMSF, 2 EDTA, and 2 EGTA, pH 7.4. To guard against proteolysis, additional protease inhibitors including leupeptin, antipain, and aprotinin were added at a concentration of 5 μg/ml. Cells were centrifuged at 1000xg to pellet cell debris and then at 14,000xg to pellet nuclear extract. Supernatants from the 14,000xg spin were used for protein analysis quantitation using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Membrane protein (500 μg) was detergent solubilized for 30 minutes with Triton X-100 extraction buffer (1% w/v Triton X-100, 150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.2% bovine serum albumin (BSA), 1 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 mmol/L benzamidine, 5 mmol/L N-ethylmaleimide, and 1 mg/ml bacitracin) and incubated on ice for 10 min. Insoluble cellular debris was removed by centrifugation at 15,000xg for 15 min. Cellular extracts were kept at 4°C throughout the solubilization process. Eight μg of a monoclonal antibody against amino acids 428-499 of rat Kv1.2 (Upstate Biotechnology, Lake Placid, New York) was added to the solubilized extract and incubated at room temperature for 2 hr on a rocking platform. Packed Protein A Sepharose beads (3 μl), preblocked with 0.2% BSA, were added and the incubation continued for an additional 2 hr at room temperature with rocking. Beads were then sedimented and washed. The bound proteins were eluted from the beads by boiling for 2 min in SDS sample buffer.

In Vitro Translation of Kv1.2 and Kv1.4

Rat Kv1.2 and Kv1.4 were synthesized in an *in vitro* translation reaction using previously described clones (Roberds & Tamkun, 1991) and the TUT-coupled reticulocyte lysate system (Promega, Madison, WI) using the manufacturers protocol.

Western Analysis of Immunopurified Kv1.2 Channel Proteins

The purified protein and *in vitro* translated proteins were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and incubated with 3% skim milk powder, 0.5% (w/v) Triton X-100, 0.1% (w/v) Tween 20 dissolved in 20 mM Tris/HCl, pH 7.4, 150 mM NaCl (TBS) for 1 hr at 22°C. Following block of the membranes, blots were incubated with monoclonal antibodies generated against amino acids 428-499 of Kv1.2 (1:10,000 dilution) and 13-37 of Kv1.4 (1:1,000 dilution). All antisera was diluted in 3% skim milk powder, 0.5% Triton X-100, 0.1% Tween 20 in TBS overnight at 4°C. Blots were washed twice with H_2O and incubated with peroxidase-conjugated goat anti-mouse IgG at a 1:2000 dilution for 90 min at 22°C. Blots were then washed two times in H_20, once in TBS + 0.1% Tween, and five additional times in H_2O and proteins identified using ECL detection methods (Amersham Life Science Inc., Arlington Heights, IL).

Solutions

The PSS used during cell isolation procedures contained in mmol/L: NaCl 120, KCl 4.2, KH_2PO_4 1.2, $MgCl_2$ 0.5, $CaCl_2$ 1.8, glucose 5.5, and HEPES 30 (pH 7.35 with NaOH). For all whole-cell voltage-clamp experiments examining Kv currents, the bath solution contained in mmol/L: NaCl 125, KCl 4.2, KH_2PO_4 1.2, $MgCl_2$ 2.3, D-glucose

5.5, TEA 10.0, and HEPES 30 (pH 7.4 with NaOH). The pipette solution for the whole-cell experiments contained in mmol/L: KCl 140, $MgCl_2$ 0.5, EGTA 1.0, ATP (magnesium salt) 5.0, and HEPES 5.0 (pH 7.2 with KOH). Dendrotoxin and mast cell degranulating peptide were obtained from Alomone Labs Inc. (Jerusalem, Israel). All other chemicals were obtained from Sigma Chemical Co.

Statistics

Results are expressed as mean ± SEM. Statistical significance was evaluated using repeated measures ANOVA and Student's T-test for unpaired data. Differences were considered significant at $P<0.05$. Membrane currents were measured from the zero current level and normalized to cell capacitance.

Results

Electrophysiological Characterization of Kv Current in Rat Renal Resistance Arterioles

Whole cell patch clamp experiments on isolated renal arteriole vascular smooth muscle cells revealed two components of Kv current. Kv current was measured in the presence of TEA (10 mmol/L) and zero external calcium. Figure 2-4A shows a representative example of current recordings following membrane depolarization from a holding potential of -80 mV. Within the range of -40 to 0 mV the evoked response is that of a slowly activating, non-inactivating, delayed rectifier-like K^+ current which we refer to as the sustained component of Kv current. Further depolarization elicited a second transient component of Kv current which activated and inactivated rapidly. This current waveform was present in every cell examined (n=65). Similar currents were recorded when ChTX (100 nmol/L) replaced the TEA or when it was applied in the presence of

TEA (data not shown). The mean current-voltage (I-V) relationship for both the transient and sustained components of Kv current are plotted in Figure 2-4B (n=11). The amplitude of the peak current component was measured immediately following the decay of the capacitive transient whereas the amplitude of the sustained current component was recorded at the end of the voltage pulse. Interestingly, compared to the sustained current component, the amplitude of the transient component of Kv current increased much more steeply with increasing depolarization. The nonlinearity of both I-V curves is indicative of outward rectification. This was further supported by the smaller than expected ratio of tail current amplitudes at -40 mV (observed ratio, 0.15; calculated ratio, 0.25). To verify that K^+ was the major charge carrier of the outward current, external $[K^+]$ was varied and tail currents examined using a double-pulse protocol. The average tail current reversal potential closely followed the predicted E_k values (data not shown).

The voltage dependence of activation and inactivation were measured and the mean data is displayed in Figure 2-4C and D, respectively. Steady-state activation was determined from the ratios of the tail currents recorded at -40 mV. The data were best fit by a single Boltzmann function and the membrane potential at which one-half ($V_{1/2}$) activation occurs was calculated to be -26.5 ± 1.8 mV with a slope factor (k) of 8.9 ± 1.5 mV (n=9). The voltage dependence of steady state inactivation was determined using a standard double pulse voltage clamp protocol. The conditioning pulse (potential reported on the abscissa of Figure 2-4D) was followed by a 2 second test pulse to +80 mV to assess open channel availability. The values for the transient and sustained current components were measured at the start and end of the test pulse and both were fit with a

single Boltzmann function. Smirnov and Aaronson (1992a) have suggested that the transient and sustained components of the TEA-insensitive Kv current in human messenteric arteries could be separated based on inactivation properties. However, in this case there was no difference between the midpoints or slopes of the two inactivation curves (transient component: $V_{1/2}$ = -38.8 ± 0.96, k=-8.9 ± 0.9 mV; sustained component: $V_{1/2}$ = -42.7 ± 3.1, k= -7.8 ± 2.7 mV, n=11).

Additional experiments were performed to separate the two components of Kv current. The rate of recovery from inactivation was examined for both the transient and sustained components of Kv current and the results are shown in Figure 2-5. A double pulse protocol was used and a representative response is displayed in Figure 2-5A. A 10 sec depolarizing voltage step to + 80 mV was used to induce inactivation. This was followed by a test pulse to +80 mV for 2 sec. The time course of recovery was assessed by varying the interpulse interval and plotting this against the normalized current. The results for the transient and sustained current components are shown in Figure 2-5B and C, respectively. The mean values for both components were best fit using bi-exponential functions each with a fast (milliseconds) and slow (several seconds) component of recovery. This is consistent with reports of recovery from N-type (fast) and C-type (slow) inactivation (Zagotta & Aldrich, 1990; Baukrowitz & Yellen, 1995). The time constants for removal of inactivation determined from these fits were not significantly different between the transient (τ_1= 19.0 ± 7.9 msec, τ_2 = 2.47 ± 1.4 sec, n=5) and sustained current components (τ_1= 14.3 ± 6.9 msec, τ_2 = 2.45 ± 1.7 sec, n=5). This suggests that the two

components of Kv current could not be separated into two populations of channels based on their inactivation properties.

Connor and Stevens (1971) first describe a transient K^+ current, termed the A-current, which activated and inactivated in a voltage range that was negative to that of delayed rectifier K^+ currents. This current has been described in vascular smooth muscle and successfully isolated from delayed rectifier K^+ currents by altering the holding potential (Beech & Bolton, 1989; Clapp & Gurney, 1991). We also tested the effect of holding potential on Kv current in vascular smooth muscle cells isolated from rat renal resistance vessels. Representative examples showing a family of currents elicited during step depolarizations from a holding potential of -80, -60, and -40 mV are shown in Figure 2-5D, E, and F, respectively. Altering the holding potential did not provide separation of the transient and sustained components of Kv current. Additional experiments employing a number of prepulse voltage protocols designed to isolate the transient or sustained current components also failed to separate the two current components (data not shown).

Pharmacological Characterization of Kv Current

4-AP is a well known inhibitor of voltage dependent K^+ channels. Differential sensitivity to 4-AP has been used to identify dissimilar Kv current components (Rudy, 1988). In our experiments, the bath application of 4-AP blocked Kv current in a dose-dependent manner. Figure 2-6 shows the dose response curve for the peak and sustained components of Kv current. Data was collected before and during drug application using successive 1 sec step depolarizations from a holding potential of -80 mV to + 80 mV with a 20 sec interval between pulses. The inset shows a representative example of the current recorded in the presence of 0, 1, and 30 mmol/L 4-AP. Data for both current components

were best fit with a single exponential function. The degree of inhibition at the maximal drug concentration was greater for the transient component (69% of control) compared to the sustained component (48% of control). However, the IC_{50} values for the two curves generated from the mean data were not significantly different (transient, ~300 μmol/L, sustained, ~200 μmol/L, n=5). This suggests that the two components of Kv current cannot be separated based on sensitivity to 4-AP.

We next examined the blocking effects of the K^+ channel toxins α-dendrotoxin (αDTX) and mast cell degranulating peptide (MCDP). αDTX, a peptide isolated from green mamba snake venom, is known to block three Shaker Kv channels (Kv1.1, Kv1.2, Kv1.6, K_d< 20 nmol/L) (Harvey, 1997). Figure 2-7A shows a representative example of the effects of 50 nmol/L αDTX on Kv current at a membrane potential of +80 mV. The toxin inhibited the current without significantly changing the shape of the current trace. This is reflected in Figure 2-7B which shows that αDTX (50 nmol/L) had similar effects on the transient and sustained current components. The average degree of inhibition was 30.8 ± 6.9% and 30.4 ± 5.8%, respectively (n=4). Increasing the concentration of αDTX to 100 nmol/L had no additional effect on the Kv current. MCDP inhibits delayed rectifier type Kv channels (Kv1.1, Kv1.2, Kv1.6) (Grissmer *et al.*, 1994). Figure 2-7C shows an example of the inhibitory effects of 1 μmol/L MCDP on Kv current elicited during a voltage step to +80 mV. The mean data is summarized in panel 2-7D. MCDP (1 μmol/L) significantly reduced the Kv current for both the transient and sustained components of Kv current but there was no significant difference in the percent inhibition between the two (transient; 49.1 ± 5.2%, sustained; 27.6 ± 15.1%,n=4).

Characterization of Kv Channels in Rat Renal Resistance Vessels

Studies have demonstrated the presence of mRNA for multiple Kv channels in rat vascular smooth muscle (Roberds & Tamkun, 1991). However, the presence of mRNA provides information on the gene expression of these K^+ channel subunits and does not confirm the presence of channel protein (Hales & Tyndale, 1994). Pharmacological evidence showed that both the transient and sustained components of Kv current were TEA-insensitive and were blocked, at least partially, by 4-AP, αDTX, and MCDP. The inability to separate a distinct transient current component from that of the sustained current suggested that heteromultimeric association may be occurring. This profile limited the number Kv channel proteins which may underlie the current and provided the framework for our initial protein screening strategy. Monoclonal Antibodies specific for the Kv1.2 channel (anti-Kv1.2) were used to immunoprecipitate Kv1.2 and associated proteins in rat renal arterioles. Anti-Kv1.2 is shown to be specific for Kv1.2 (Fig. 2-8 left panel) since it immunoblots Kv1.2 *in vitro* translated core protein (~55 kDa) as well as processed forms of Kv1.2 translated in the presence of canine microsomal membranes (~80 kDa), but not a negative control or Kv1.4 *in vitro* translated protein. Immunoprecipitation of Kv1.2 followed by immunoblotting with anti-Kv1.2 did recognize a processed form of Kv1.2 in rat renal arterioles of ~80 kDa. To determine whether Kv1.2 heteromultimerizes with other shaker-type channels, immunoprecipitations with Kv1.2 were then immunoblotted with antibodies specific for Kv1.4 (anti-Kv1.4, right panel). Western blotting with a monoclonal antibody against Kv1.4 recognized a Kv1.4 band of ~96 kDa suggestive that Kv1.2 and Kv1.4

heteromultimerize in rat renal arterioles. Anti-Kv1.2 and anti-Kv1.4 were specific and did not recognize a band in sham *in vitro* translated core proteins (control lanes).

Discussion

This study characterizes the biophysical and pharmacological properties of the Kv current in vascular smooth muscle cells isolated from rat renal resistance arterioles. We have identified two Kv channel proteins, Kv1.4 and Kv1.2, which may underlie the observed Kv current and provide the first direct *in vivo* evidence for heteromeric association of Kv channel α-subunits in vascular smooth muscle.

First inspection of the K^+ current, elicited by step depolarizations, suggested the presence of two overlapping voltage-dependent currents. A transient current component which activated at depolarized potentials appeared superimposed on a non-inactivating delayed rectifier-like current component (Fig. 2-4A). This was similar to that reported by Smirnov and Aaronson (1992a) in human mesenteric arteries and by Gordienko *et al.* (1994) in rat renal resistance arterioles. Both of these reports described a transient current which may be classified as a "high-threshold subclass" of A-current (Rudy, 1988) and which overlapped a slow activating, sustained component. However, unlike these reports we were unable to separate the transient and sustained components based on kinetic or pharmacological differences. Steady-state inactivation (Fig. 2-4D), rate of recovery from inactivation (Fig. 2-5A-C), and sensitivity to 4-AP (Fig.2-6) were similar for both components. In addition unlike other A-type currents described in smooth muscle, we were unable to isolate the transient current by varying the holding potential (Beech & Bolton, 1989; Clapp & Gurney, 1991).

When expressed in heterologous systems, Kv1.4 homomeric channels are known to produce a TEA-insensitive transient current which recovers slowly from inactivation and is sensitive to 4-AP (Stuhmer *et al.*, 1989; Ruppersberg *et al.*, 1990). While this current is similar to the transient component which we observed in native vascular smooth muscle cells, differences in the voltage range of activation and recovery from inactivation suggest that this channel alone may not account for the transient component of Kv current. Furthermore, αDTX and MCDP which are toxins specific for the delayed rectifier-like currents Kv1.1, Kv1.2, and Kv1.6 blocked both the transient and sustained components of Kv current equally and incompletely. Of these three gene products, only Kv1.2 encodes for a channel which produces a TEA-insensitive current suggesting that this protein may also contribute to the subunit composition of Kv channel complexes in rat renal arterioles. Western Blot analysis of immunopurified protein demonstrated the presence Kv1.4 and Kv1.2 α-subunit immunoreactive-like protein in this tissue (Fig. 2-8).

Our electrophysiological data suggested that Kv1.2 and Kv1.4 may associate in rat renal resistance vessels to form a distinct current with properties that are different from that displayed by either Kv1.2 or Kv1.4 homomers. The endogenous association of these two channel proteins was confirmed by co-immunoprecipitation from rat renal arteriole homogenate (Fig. 2-8). This biochemical strategy has been used *in vivo* to demonstrate that Kv channels (including Kv1.2 and Kv1.4) assemble as heteromultimers in rat brain (Sheng *et al.*, 1993; Wang *et al.*, 1993; Shamotienko *et al.*, 1997). In addition, the heteromultimeric assembly of Kv1.4 and Kv1.2 has been study *in vitro*

using *Xenopus* oocytes (Po *et al.*, 1993). Comparison of the properties between the hybrid Kv1.4/Kv1.2 complex reported by Po *et al.* (1993) to those of the native Kv current in rat renal resistance arteries reported herein reveals a strong similarity. Both currents are TEA-insensitive, highly sensitive to 4-AP, exhibit fast inactivation, rapid recovery from inactivation, and display the same relationship between steady-state inactivation and membrane potential. This parallel provides further evidence that Kv1.4 and Kv1.2 α-subunits determine the subunit composition of at least one Kv channel complex in rat renal arterioles. Recent evidence demonstrates that the presence of a single inactivating Kv1.4 subunit confers N-type inactivation on heteromultimeric channels (Lee *et al.*, 1996). In addition, reports demonstrate that the biophysical properties of heteromultimeric channels are quantitatively more similar to the most abundant subunit (Hopkins *et al.*, 1994). However, it is important to note that the difficulty in separating the transient and sustained components of Kv current does not exclude the possibility of additional Kv channel complexes or the participation of kinetic altering β-subunits. Indeed, there is evidence for the presence of Kv channel β-subunits in vascular smooth muscle (mRNA, Morales *et al.*, 1995; protein, S.K. England unpublished observations in renal arterioles). The equal but incomplete blockade of both the transient and sustained components by αDTX and MCDP may signal the presence of additional components which we were unable to isolate with the methods presented here. Alternatively, recent evidence suggests that high affinity binding of αDTX to Kv channels requires that all four individual subunits of a heterotetramer posses an αDTX binding site (Tytgat *et al.*, 1995). This would not be satisfied by a heterotetramer

containing Kv1.4 α-subunits. Ultimately, the contribution of Kv channel subunits and multimeric complexes to native current requires specific approaches such as the use of antisense strategies or gene knockout experiments.

The physiological role of a transient current in vascular smooth muscle is an unanswered question and requires further study. The outward rectification and high voltage threshold for activation suggests a role for the transient current in limiting membrane depolarization. In small resistance arteries which respond to vasoconstricting stimuli with graded depolarizations, the transient current would activate rapidly and carry a large hyperpolarizing current. This may be important during times of ischemic insult or in disease states such as hypertension which are characterized by increased vasoconstriction. Recently, Roeper *et al.* (1997) have shown that phosphorylation of Kv1.4 by Ca^{2+}/calmodulin dependent protein kinase leads to a slowing of inactivation gating and accelerated recovery from N-type inactivation. In smooth muscle this would result in an increased availability of hyperpolarizing current during times of elevated intracellular Ca^{2+} and may represent a modulatory mechanism for the transient current during excitation-contraction coupling in health and disease states.

In summary, we have provided electrophysiological, pharmacological, and biochemical evidence for the association of Kv1.2 and Kv1.4 α-subunits in rat renal resistance arteries. This is the first direct *in vivo* evidence for the existence of heteromultimeric Kv channels in vascular smooth muscle. This work suggests that similar to neuronal and cardiac organ systems, vascular smooth muscle also use hetero-oligomeric assembly to generate K^+ channel diversity.

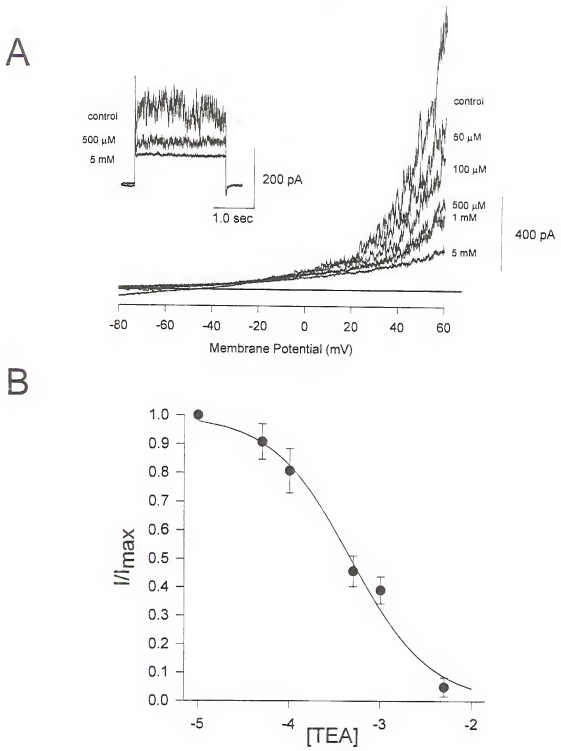

Figure 2-1. Effect of tetraethylammonium (TEA) on K_{ca} current. (A) Representative example of 4 sec. Ramp depolarization from a holding potential of -80 mv to +80 mV in the presence of 4-AP and increasing concentrations of TEA. The inset shows a representative example of step depolarizations from -80 mV to +40 mV in the presence of 0, 500 µmol/L and 5 mmol/L TEA. (B) Dose response curve for TEA as measured from step depolarizations to +40 mV (IC_{50} = 683.1 µmol/L, n=4). Current is displayed as a fraction of control (I_{max}).

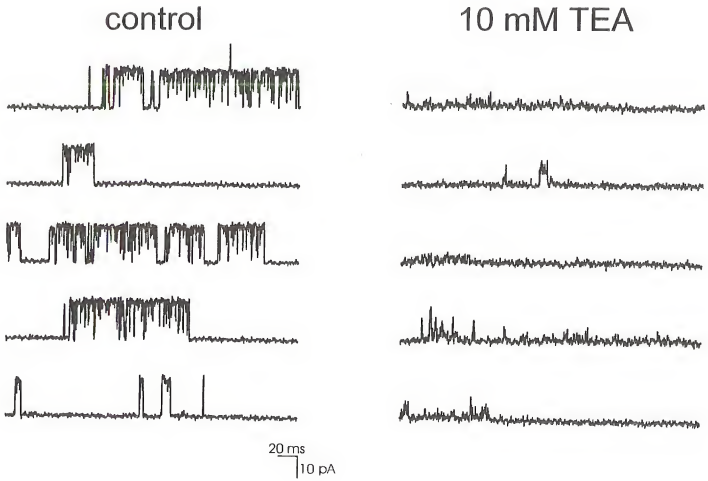

Figure 2-2. Effect of tetraethylammonium (TEA) on single channel K_{Ca} currents. Shown are single channel recordings of K_{Ca} current at +40 mV in the absence and presence of 10 mmol/L TEA.

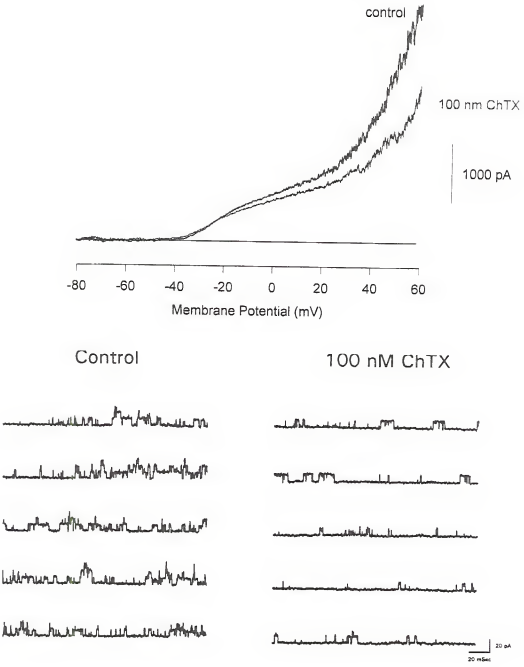

Figure 2-3. Effect of charybdotoxin (ChTX) on calcium activated K^+ current. (A) Representative example of a 4 sec. ramp depolarization from a holding potential of -80 mV to +80 mV in the absence and presence of 100 nmol/L ChTX. (B) Single channel recordings of K_{Ca} current at +40 mV using the cell attached patch technique in the absence and presence of ChTX. The toxin inhibits most but not all single channel activity.

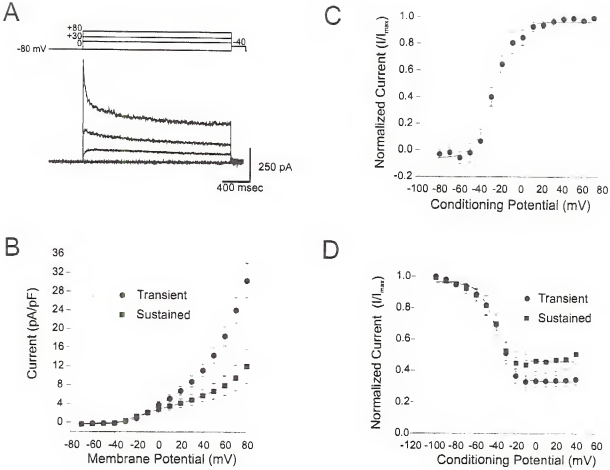

Figure 2-4. Electrophysiological characteristics of the Kv current in VSM cells isolated from rat renal resistance arteries. (A) Representative recordings of current elicited during voltage-step depolarizations in zero external Ca^{2+} and 5 mM TEA. (B) Graph showing the mean current-voltage relationship for both the peak and sustained component of Kv current (n=11). Current amplitude is normalized to each cell's capacitance. (C) Voltage dependence of Kv current activation as determined from the magnitude of the tail current (mean ± SE, n=9). (D) Voltage dependence of Kv current inactivation determined using a double pulse protocol (mean ± SE, n=11).

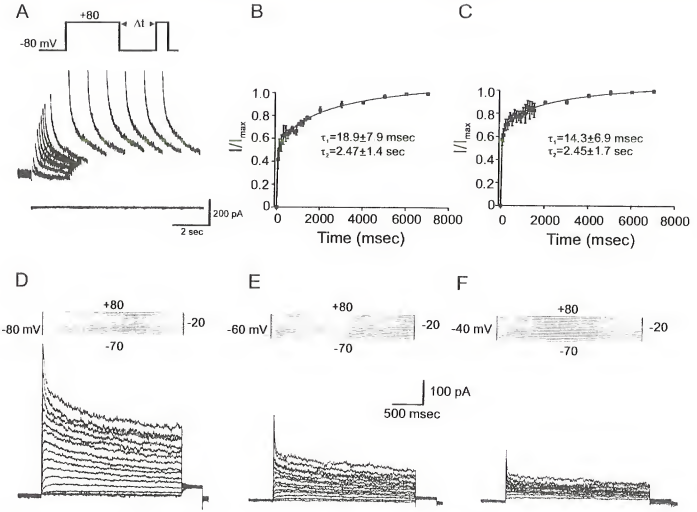

Figure2-5. Time course of recovery from inactivation and the effect of holding potential on Kv current. (A) Representative example showing recovery of current following inactivation. The experimental protocol is shown above. (B,C) Graph showing the mean time course of recovery from inactivation for the transient (B) and sustained (C) component. The data was best fit by a bi-exponential function. The two time constants for each fit are reported on the graph (mean SE, n=5). (D,E,F) Within the same cell, representative examples of a family of currents evoked from a holding potential of -80, -60, and -40 mV respectively. The voltage pulse protocol is shown above.

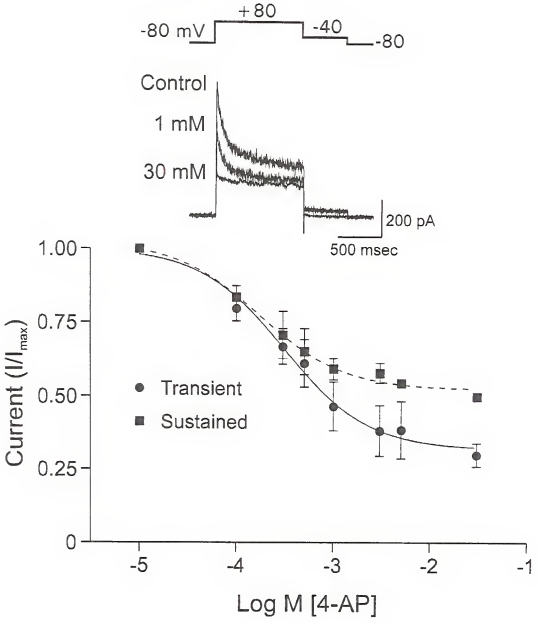

Figure 2-6. Mean dose-response relations showing the effects of 4-aminopyridine (4-AP) on Kv current. I/I_{max}, percentage of current in the presence of 4-AP as a fraction of its control value. Graph shows the effect of 4-AP on both peak and sustained components of current (mean ± SE, n = 5). Currents were measured at + 80 mV. Top shows a representative recording of Kv current measured at + 80 mV from a holding potential of -80 mV in the presence of 1 and 30 mmol/L 4-AP.

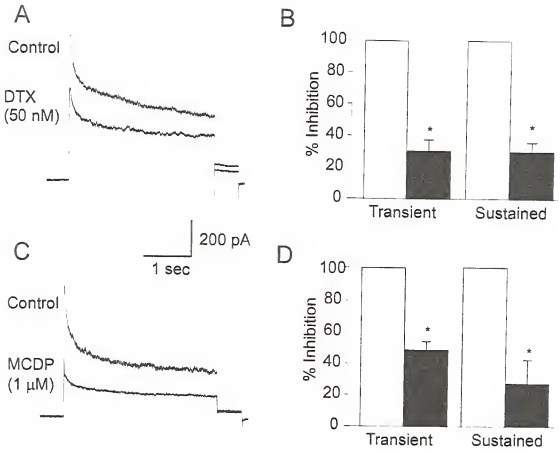

Figure 2-7. Effect of αDTX and MCDP on Kv current. (A) Representative recording of Kv current in response to a step depolarization from -80 mV to +80 mV in the absence and presence of αDTX (50 nmol/L). (B) Graph showing the % inhibition by αDTX (50 nmol/L) of both the peak and sustained components of Kv current (mean ± SE, n=4). (C) Representative recording of Kv current in response to a step depolarization from -80 mV to +80 mV in the absence and presence of MCDP (1μmol/L). (D) Graph showing the % inhibition by MCDP (1μmol/L) of both the peak and sustained components of Kv current (mean ± SE, n=4).

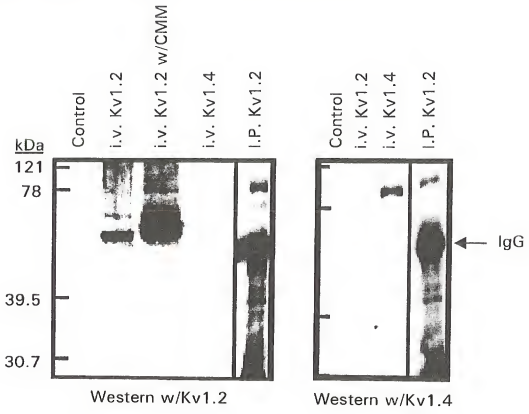

Figure 2-8. Co-immunoprecipitation of Kv1.2 and Kv1.4 from rat renal arterioles. Membranes isolated from rat renal arterioles were immunoprecipitated with anti-Kv1.2 antibody and size fractionated by SDS-PAGE. Samples were transferred to nitrocellulose and probed with anti-Kv1.2 or anti-Kv1.4 antibodies and detected using ECL. A band corresponding to a processed form of Kv1.2 of ~80 kDa (left panel) was detected by the anti-Kv1.2 antibody (I.P. Kv1.2) whereas a band of ~96 kDa was recognized by the Kv1.4 antibody (I.P. Kv1.2, right panel). Both antibodies were isoform specific and only recognized their corresponding in vitro translated product on immunoblots (lanes marked i.v. Kv1.2, i.v. Kv1.2 w/CMM, i.v. Kv1.4). Neither antibody recognized sham in vitro translated proteins (control lanes). The position of the IgG antibody from the immunoprecipitation as detected with goat anti-mouse secondary antibody is indicated by the arrow.

CHAPTER 3
ALTERATIONS IN RAT INTERLOBAR ARTERY MEMBRANE POTENTIAL AND K^+ CHANNELS IN GENETIC AND NONGENETIC HYPERTENSION

Introduction

The etiology of systemic hypertension involves a gradual and sustained increase in total peripheral resistance (Bohr & Webb, 1988). A number of anatomical, biochemical or biophysical mechanisms may underlie this phenomenon (Khalil *et al.*, 1989). Anatomically, rarefaction of the vascular beds and blood vessel hypertrophy occurs in many hypertensive models. Biochemically, alterations in Na^+, K^+-ATPase, Ca^{2+}-ATPase and Na^+-Ca^{2+} exchange activity have been reported in blood vessels and isolated cell preparations from various models of hypertension. Biophysical changes that may occur in hypertension involve changes in ion channel function thereby causing changes in vascular reactivity. Vascular smooth muscle contains at least two different Ca^{2+} channels, at least four different potassium channels, non-selective cation channels, and Ca^{2+}-activated Cl^- channels (Pacaud *et al.*, 1989b; Nelson *et al.*, 1990b; Lamb *et al.*, 1994; Nelson & Quayle, 1995). Therefore, alterations in ion channel function of any of these channels could potentially change the contractile state of vascular smooth muscle thereby altering blood pressure.

The use of fluorescent Ca^{2+} indicators and electrophysiological techniques have enabled studies of hypertension at the single cell level. However to date, the data from

various models have led to differing hypotheses regarding the roles of ion channels in the regulation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and in the etiology of hypertension. For example, when SHR aortic smooth muscle cells were loaded with fura-2, basal $[Ca^{2+}]_i$ was increased when compared to WKY cells (Sugiyama *et al.*, 1986). Furthermore, vasopressin (AVP) and angiotensin II (Ang II)-stimulated increases in $[Ca^{2+}]_i$ were greater in SHR aortic cells when compared to WKY cells (Nabika *et al.*, 1985). Conversely, Storm *et al.* (1992) and England *et al.* (1993) show no change in basal $[Ca^{2+}]_i$ levels in hypertension. Using conventional microelectrodes, it was reported that the ouabain-insensitive component of resting membrane potential is 10-15 mV more depolarized in SHR blood vessels than WKY vessels (Hermsmeyer, 1976). However, other laboratories report that no change in resting membrane potential exists in hypertensive models (Lamb & Webb, 1989; Steikel, 1989; Rusch *et al.*, 1992). Recently, Rusch *et al.* (1992) and England *et al.* (1993) demonstrated that SHR aortic cells have more Ca^{2+}-activated K^+ current (K_{Ca}) than WKY cells. However, no studies to date have shown alterations in Kv in hypertensive models. To date, the fluorescence and electrophysiological studies performed in various models of hypertension have been obtained from larger conduit arteries which play a less significant role in setting peripheral vascular resistance than blood vessels from the kidney or mesentery. Studies on clinically relevant vessels from the renal vasculature should provide useful information on the electrophysiological differences in hypertension at the single cell level. Here we present data which for the first time demonstrates that alterations in Kv channel activity, membrane potential, and $[Ca^{2+}]_i$ exist in small vessels of the genetic and nongenetic hypertensive renal vasculature.

Methods

Animal Preparation

WKY, Sprague-Dawley (SD), and SHRs were bought from Charles River Breeders (Boston, MA). Control SD rats were made hypertensive by implanting one 50 mm long silastic tube (Dow Corning), containing deoxycorticosterone acetate (DOCA, 100 mg/Kg), subcutaneously on the dorsal side of the rats. Rats were unilaterally nephrectomized and given 0.15 mmol/L NaCl to drink as their sole drinking fluid. Control blood pressures are taken two weeks before the above procedure and weekly thereafter. Hypertension developed in 4-6 weeks after DOCA/salt/nephrectomy regime. Adult male rats were used at the age of 15 weeks. The mean systolic blood pressure of WKY rats was140 ± 4 mm Hg (n=15) and was significantly greater in the SHRs, 195 ± 11 mm Hg (n=15, $p<0.01$). Similar results were obtained for the SD and DOCA hypertensive rats (SD: 115 ± 7 mm Hg; DOCA: 165 ± 10 mm Hg, (n=16, $p<0.01$). WKY and SHR rats are used as the control and experimental model for genetic hypertension; the SD and DOCA hypertensive rats are used as the control and experimental model for nongenetic hypertension.

Electrophysiological Measurements

Single smooth muscle cells from WKY, SHR, SD, and DOCA hypertensive interlobar artery were enzymatically dissociated using previously described methods. Care was taken to minimize trauma to either arterial preparation. Cells from normotensive and hypertensive animals were made on the same day using the same procedure thereby ruling out nonspecific membrane effects that were due to the cell

isolation technique. All experiments were conducted blind thereby minimizing bias in the results. However, there is a possibility that the connective tissue of the arterial wall is altered in hypertension (Khalil *et al.*, 1989) causing the effectiveness of the digestive enzymes to be altered. Thus, smooth muscle cells isolated from the vessels of hypertensive rats may have been in the presence of the enzymatic digestion cocktail for longer periods of time than control, possibly altering sarcolemma proteins. Although difficult to test, efforts were made to reduce the possible effects of variable digestion in the tissues by examining reversibility of an angiotensin II (Ang II, 100 nmol/L) induced contraction of isolated single smooth muscle cells. Only batches of cells that showed contraction and relaxation to Ang II were used.

Single cells were voltage clamped and membrane currents were measured using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). Patch pipettes for whole-cell patch clamp recordings were made from borosilicate glass capillaries and had resistances of 1-3 MΩ. Voltage clamp command potentials were applied to the cells and membrane currents were recorded using an Axopatch-1D patch clamp amplifier. Membrane current was monitored on a digital oscilloscope, digitized on-line (0.5-2.0 kHz), and stored on a computer. Resting membrane potentials were measured in the I=0 position of the Axopatch 1-D. Input resistances were determined in current clamp by applying a 1-5 pA hyperpolarizing current pulse and examining the change in membrane potential. Any cell that did not have a seal resistance of greater than 5 GΩ was not used. Series resistance was in the range of 2-6 MΩ and was compensated for by approximately 80% using the Axopatch-1D. Junction potentials were zeroed at

the beginning of each experiment with Axopatch-1D. At the end of each experiment, no change in the nulled junction potential occurred. Data analysis was performed with pCLAMP 5.5.1 and 6.0 software (Axon Instruments). All experiments were performed at room temperature and data illustrated in the figures were from different batches of cells isolated from different animals.

Measurement of Intracellular Ca^{2+}

The Ca^{2+} indicator indo-1 (pentapotassium salt, 100 μmol/L) was included in the patch pipette solution and dialyzed into the cell as previously described (Gelband & Hume, 1995). Background autofluorescence from the same cell was measured before gaining access to the cell interior and subtracted from all fluorescence measurements. Sufficient loading had taken place 5-10 minutes after access to the cell interior. A 10 μm diameter of the cell was irradiated with ultraviolet light at a wavelength of 340 nm with a mercury lamp. The light emitted from the cell was collected using an epifluorescence microscope (Nikon) and measured at 400 and 500 nm by means of a microfluorometer and matched photomultiplier tubes. Changes in $[Ca^{2+}]_i$ were calibrated using the equation of Grynkiewicz *et al*, 1985*)*:

$$[Ca^{2+}]_i = K_d[R-R_{min}]/R_{max}-R](S_{f2}/S_{b2})$$

where K_d is the dissociation constant of the Ca^{2+}-Indo-1 complex; R is the F_{400}/F_{500} fluorescence ratio; R_{min} and R_{max} are the ratios measured by the addition of the Ca^{2+} ionophore ionomycin (10 μmol/L) to Ca^{2+} free (10 mmol/L EGTA) solution and Ca^{2+}containing solution respectively; and S_{f2}/S_{b2} is the ratio of the 500 NM fluorescence signal in Ca^{2+} free and Ca^{2+} containing solutions.

Solutions

The Krebs solution contained (in mmol/L): 120 NaCl, 25 $NaHCO_3$, 4.8 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 D-glucose. For all whole-cell voltage clamp experiments examining outward currents, the bath solution contained (in mmol/L): 130 NaCl, 10 $NaHCO_3$, 4.2 KCl, 1.2 KH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 5.5 D-glucose, and 10 HEPES, (pH 7.4 with NaOH). The pipette solution contained (in mmol/L): 140 KCl, 5 ATP (potassium salt); 10 HEPES, (pH 7.2, with KOH). ChTX was obtained from Peninsula Laboratories, Inc. and the stock was 0.1 M in 150 mmol/L NaCl. All other chemicals were from Sigma Chemical Company.

Statistics

Results are expressed as mean ± S.E.M. Statistical significance was evaluated using Student's *t* test for unpaired observations. Differences were considered significant at $p<0.05$. *n* corresponds to the number of cells examined. Membrane currents were measured from the zero current level and normalized to cell capacitance.

RESULTS

Electrical Properties of Single Rat Interlobar Artery Cells

Table 3-1 illustrates the electrical properties of isolated WKY, SD, SHR and DOCA hypertensive interlobar artery cells. In 15-16 cells from 9 different preparations, a significant difference was observed in the resting membrane potential of the cells. WKY, SD, SHR and DOCA hypertensive cells had an average resting membrane potential of -52 ± 2.3, -50 ± 3.8, -32 ± 3.8* and -36 ± 2.5 mV*, respectively (n=15 or 16, *$p<0.01$). All cells had similar input resistances and membrane capacitances (Table 3-1). One

explanation of these results is that the altered contractile state of the hypertensive interlobar artery may be due to alterations in K^+ permeability of the hypertensive single smooth muscle cell.

Ionic Currents in WKY, SD, SHR and DOCA Hypertensive Cells

It has been reported that K_{Ca} was increased in hypertension, (Rusch *et al.*, 1992; England *et al.*, 1993) but to date no evidence for changes in Kv have been demonstrated in hypertensive models. Figure 3-1 shows pharmacological isolation of Kv and K_{Ca} from WKY and SHR cells. The use of pharmacological agents to isolate these two components of K^+ current was based on experimental results obtained from the canine renal artery (Gelband & Hume, 1995). ChTX (100 nmol/L) and niflumic acid (100 mmol/L) were used to isolate Kv; 4-AP (10 mmol/L) and niflumic acid (100 mmol/L) were used to isolate K_{Ca}; and 5 mmol/L ATP was present in the recording pipette to inhibit K_{ATP}. Figure 3-1A (upper panel), shows representative ramp depolarizations from a WKY and an SHR cell in the presence of 4-AP and niflumic acid. Under these conditions Kv was significantly reduced, yet K_{Ca} was present. Figure 3-1A (bottom panel) shows the mean current-voltage relationship obtained for K_{Ca} during voltage step depolarizations (n=7). In 7 experiments, K_{Ca} in SHR cells was significantly increased when compared to control ($P<0.01$, Table 2). Figure 3-1B illustrates the nature of Kv in WKY and SHR cells in the presence of ChTX and niflumic acid. The upper panel shows representative ramp depolarizations and under these conditions K_{Ca} was nearly abolished, yet Kv was present. The lower panel shows the mean current-voltage relationship obtained for Kv during voltage step depolarizations (n=7). In 7 cells tested, Kv was decreased in SHR cells when compared to WKY cells during voltage step depolarizations

($P<0.01$). Table 3-2 illustrates mean current density values for K_{Ca} and Kv in WKY and SHR cells at membrane potentials of 0 and 40 mV.

Similar results were obtained when Kv and K_{Ca} were isolated in control and non-genetic hypertensive cells. Figure 3-2 illustrates the characteristics of Kv in SD and DOCA hypertensive cells. During voltage step depolarizations in the presence of charybdotoxin and niflumic acid (Panel A), Kv in DOCA hypertensive cells is less than control. During a voltage ramp depolarization (Fig. 3-2B), the DOCA hypertensive cells possessed a decreased Kv when compared to control. In another set of experiments, the nature of K_{Ca} in SD and DOCA hypertensive cells was investigated (Fig. 3-3). Panel A shows representative traces of step depolarizations from a SD and a DOCA hypertensive cell in the presence of 4-AP and niflumic acid. K_{Ca} was significantly increased in DOCA hypertensive cells when compared to control ($n=7$, $P<0.01$, Table 3-2). Panel B is a representative trace of K_{Ca} during voltage ramp depolarizations in both cell types. Again it shows that the DOCA hypertensive K_{Ca} was greater when compared to control. Figure 3-4A and B illustrate mean current voltage relationships for Kv and K_{Ca} during voltage step depolarizations in SD and DOCA hypertensive cells ($n=7$, $P<0.01$). The DOCA hypertensive mean Kv current-voltage relationship was right shifted and activated at more positive potentials than control (Panel A). However, the DOCA hypertensive mean K_{Ca} current-voltage relationship was left shifted and activated at more negative potentials than control (Panel B). Table 3-2 illustrates mean current density values at 0 and 40 mV for K_{Ca} and Kv in SD and DOCA cells. These biophysical characteristics may be of some importance since K^+ current has been suggested to play a role in regulating resting membrane potential of vascular smooth muscle cells (Fleischmann *et al.*, 1993; Gelband

& Hume, 1995).

Regulation of Intracellular $[Ca^{2+}]$ in WKY, SD, SHR and DOCA Hypertensive Cells

As illustrated in Table 3-1, the resting membrane potential of SHR and DOCA hypertensive artery cells was more depolarized than control cells. Since $[Ca^{2+}]_i$ has been suggested to inhibit K_v channels and regulate membrane potential, (Gelband & Hume, 1995) a separate group of experiments were performed to investigate the regulation $[Ca^{2+}]_i$ in single WKY, SD, SHR and DOCA hypertensive interlobar artery cells. Figure 3-5 shows that resting levels of $[Ca^{2+}]_i$ was significantly elevated in the hypertensive cells when compared to control (WKY: 92 ± 7 nmol/L; SHR: 125 ± 9 nmol/L*; SD: 89 ± 7 nmol/L; DOCA: 113 ± 11 nmol/L*, n=6, $p<0.05$). Since circulating Ang II is a potent renal vasoconstrictor and its circulating concentration is increased in various forms of hypertension, (Khalil *et al.*, 1989) we investigated the effects of Ang II on peak $[Ca^{2+}]_i$. Application of Ang II (100 nmol/L) significantly increased peak $[Ca^{2+}]_i$ over basal levels in each cell type (n=6). This occurred within 5 sec of Ang II application. A significant difference was also observed in the Ang II-stimulated increase in peak $[Ca^{2+}]_i$ when hypertensive cells were compared to control cells (WKY: 450 ± 25 nmol/L; SHR: 665 ± 30 nmol/L*; SD: 369 ± 31 nmol/L; DOCA: 595 ± 20 nmol/L*, n=6, $p<0.05$). No difference was observed in the time course of the Ang II effect in any cell type. When combined with the results of Figures 3-1 thru 3-4, these data support the hypothesis that $[Ca^{2+}]_i$ may modulate resting membrane potential by altering Kv (Gelband & Hume, 1995) and/or K_{Ca} current (Brayden & Nelson, 1992).

DISCUSSION

Alterations in ion channel activity have been associated with an increased vascular reactivity in hypertension (Khalil *et al.*, 1989; Steikel, 1989). Specifically, depolarization of the resting membrane potential, (Hermsmeyer, 1976; Tomobe *et al.*, 1991; Van-de *et al.*, 1992) and increases in Ca^{2-} current,(Rusch & Hermsmeyer, 1988; Ohya *et al.*, 1993; Wilde *et al.*, 1994) K_{Ca} (Rusch *et al.*, 1992;England *et al.*, 1993) and $[Ca^{2+}]_i$ (Losse *et al.*, 1984; Sugiyama *et al.*, 1986; Bukoski, 1990; Papageorgiou & Morgan, 1991; Storm *et al.*, 1992) have been demonstrated in various forms of hypertension. The present study describes the electrophysiological properties of a physiologically relevant vascular preparation, the interlobar artery of the kidney, in hypertension. Our results suggest that there are changes observed in both K^+ current and Ca^{2+} homeostasis in a genetic and nongenetic model of hypertension. These effects may contribute to the increase in vascular reactivity observed in hypertension.

Resting Membrane Potential and Hypertension

Normotensive vascular smooth muscle cells including those isolated from the renal vasculature have resting membrane potentials between -50 and -60 mV (Harder & Hermsmeyer, 1983b; Gelband & Hume, 1992; Rusch *et al.*, 1992; Fleischmann *et al.*, 1993; Gelband & Hume, 1995). In the present study, it was shown that SHR and DOCA hypertensive interlobar artery cells have a resting membrane potential of approximately -30 mV (Table 3-1). This was significantly depolarized when compared to the control cells (approximately -50 mV). In a variety of conduit artery vascular preparations, significant changes in passive membrane electrical properties have been reported in SHR cells when compared to control (Hermsmeyer, 1976; Harder *et al.*, 1983a; Harder & Hermsmeyer,

1983b; Lamb & Webb, 1989; Tomobe *et al.*, 1991). However, there have been some reports of an unaltered resting membrane potential in vascular smooth muscle cells from the SHR and other models of hypertension (Abel *et al.*, 1981; Steikel, 1989; England *et al.*, 1993). One reason for the discrepancy in resting membrane potentials may be the site and size of the vessel itself. There is ample evidence that larger capacitance vessels, like the aorta and first branch coronary arteries, do not have similar excitation-contraction coupling properties when compared to smaller resistance vessels (Van Breemen & Saida, 1989). This suggests that small arteries (<200 μm in diameter) from physiological relevant vascular beds (e.g. renal, mesenteric and cerebral) are altered in hypertension and play a significant role in the regulation of peripheral resistance.

K^+ Channels and Hypertension

Membrane K^+ permeability is the primary initiator of vascular smooth muscle contractility (Nelson *et al.*, 1990b; Nelson & Quayle, 1995). $^{45}K^+$ efflux from conduit artery hypertensive vascular smooth muscle was increased when compared to normotensive vascular smooth muscle (Jones, 1973). To date there is no evidence that alterations in voltage dependent K^+ channels exist in any model of hypertension. Our results are the first to show that in SHR and DOCA hypertensive interlobar artery cells, Kv was significantly reduced when compared to control (Figs. 3-1 and 3-3). A significant decrease of Kv current would cause membrane depolarization and an increase in vascular smooth muscle tone. Indeed a significant depolarization of the resting membrane potential of isolated cells from SHR and DOCA hypertensive animals was observed when compared to control (Table 3-1). This would suggest that Kv channels

play a major role in the regulation of membrane potential and therefore tone in rat interlobar arteries.

It was previously demonstrated there was an increased K_{Ca} and open probability of large conductance K_{Ca} channels in SHR cells isolated from the aorta (Rusch *et al.*, 1992; England *et al.*, 1993). It was suggested that this enhanced K^+ permeability may provide a negative feedback mechanism by which an increased arterial contractility may be limited in hypertension (Rusch *et al.*, 1992). The data described in this manuscript are consistent with these conclusions. Basal $[Ca^{2+}]_i$ is increased in the SHR and DOCA hypertensive cells (Fig. 3-5), and this would lead to a greater K_{Ca} current density in the SHR and DOCA hypertensive cells (Figs. 3-1 and 3-2). However, based on the voltage-dependent properties of this channel (i.e. extremely low open probability at more negative membrane potentials), it would be difficult for this channel to be the sole channel protein that causes the increased $^{45}K^+$ efflux observed in SHR arterial smooth muscle.

$[Ca^{2+}]_i$ and Hypertension

A number of studies have attempted to compare resting levels of $[Ca^{2+}]_i$ in SHR and WKY artery preparations (Losse *et al.*, 1984; Nabika *et al.*, 1985; Sugiyama *et al.*, 1986; Erne & Hermsmeyer, 1989; Khalil *et al.*, 1989; England *et al.*, 1993; Rusch *et al.*, 1992Nelson & Quayle, 1995). These studies have shown differing results depending mainly on the age of the animal used. In preparations of SHR arteries from young animals (3 days to 5 weeks) which have not developed hypertension, resting $[Ca^{2+}]_i$ levels did not differ from the WKY (Khalil *et al.*, 1989; Storm *et al.*, 1992; Nelson & Quayle, 1995). However, Ang II or vasopressin-stimulated changes in $[Ca^{2+}]_i$ did show significant differences in young SHR rats, suggesting a change in Ca^{2+} homeostasis at an early age in

development before the onset of hypertension (Nabika *et al.*, 1985). This is consistent with our results (Figs. 3-3 to 3-5). In older rats (>8 weeks) an increase in resting levels on $[Ca^{2+}]_i$ between control and hypertensive cells was observed (Losse *et al.*, 1984; Sugiyama *et al.*, 1986). However, there are studies stating that resting levels of $[Ca^{2+}]_i$ were not altered in adult SHR vascular smooth muscle cells from conduit arteries (Storm *et al.*, 1992; England *et al.*, 1993). The present data using indo-1 fluorescence suggests that $[Ca^{2+}]_i$ was significantly greater in SHR and DOCA hypertensive interlobar artery cells than in control. This increase in resting $[Ca^{2+}]_i$ levels can be directly correlated to the changes observed in membrane potential and Kv current. Previous results in the canine renal artery (Gelband & Hume, 1995) demonstrated the sensitivity of Kv channels to increased $[Ca^{2+}]_i$ which in turn caused membrane depolarization. These data are consistent with our observations that cells from resistance blood vessels have different excitation-contraction coupling pathways and Ca^{2+} homeostatic mechanisms than cells from conduit blood vessels.

Ion Channel Control of Vascular Smooth Muscle Tone in Hypertension

Recently, a number of K^+ channels have been suggested to regulate membrane potential and tone of smooth muscle cells. It has been demonstrated that voltage dependent delayed rectifier K^+ channels are important regulators of smooth muscle resting membrane potential (Fleischmann *et al.*, 1993; Gelband & Hume, 1995). This is consistent with our observations in the interlobar artery smooth muscle cells. Our results in clinically relevant resistance vessels suggest that the inhibition of Kv and the associated membrane depolarization would be of physiological importance to the regulation of tone in normotensive and hypertensive states (Gelband & Hume, 1995).

However, other K^+ channels have also been suggested to play a role in the regulation of smooth muscle tone. Large conductance K_{Ca} channels may represent a negative feedback mechanism for cerebral arteries when pressurized (Erne & Hermsmeyer, 1989). K_{ATP} (Clapp & Gurney, 1992) and K_{Ir} channels (Quayle *et al.*, 1993) also have been implicated to play a role in regulating tone in the small vessels of cerebral, pulmonary and coronary circulation. Therefore a combination of K^+ channels or variation in K^+ channel distribution may explain why different K^+ channels are observed to regulate membrane potential in various vascular beds.

Significance of the Kidney in Hypertension

Despite years of intensive investigation, the etiology of hypertension remains unknown. Elevated blood pressure and total peripheral resistance associated with hypertension may result from increases in neural, humoral, or vasoconstrictor substances, passive or genetic structural alterations in blood vessels, or intrinsic changes in the control of vascular tone. The interlobar artery of the kidney is a significant blood vessel to study since alterations in renal blood flow are known to influence fluid volume regulation, the secretion of a number of important neurohumoral substances, and arteriolar resistance which may be involved in the etiology or maintenance of hypertension. However, direct measurements of the major ionic conductances that may underlie contraction of hypertensive rat interlobar arterial smooth muscle are lacking. This is rather remarkable given the results of earlier renal transplant studies in humans (Bianchi *et al.*, 1979; Curtis *et al.*, 1983) and animals (Dahl *et al.*, 1972) which suggested that the genetic defect in a proportion of the cases with essential hypertension is expressed in the kidney. In these studies, kidney transplants from normotensive donors

into hypertensive recipients effectively reversed many of the abnormalities associated with hypertension. In summary, the data demonstrate that the alterations in resting membrane potential observed in the SHR and DOCA hypertensive interlobar artery could be manifest through alterations in one or a combination of Kv channels or K_{Ca} channels. These alterations in ionic conductances may play a role in the etiology and/or maintenance of the hypertensive state.

Table 3-1. Electrical Properties of WKY, SD, SHR, and DOCA Rat Interlobar Artery Cells

Cell Type	*n*	V_m(mV)	R_{input}(GΩ)	C_m(pF)
WKY	15	-52±2.3	5.1±1.2	25±2.6
SHR	15	-32±3.8*	5.6±2.3	26±1.8
SD	16	-50±3.6	6.3±2.5	29±2.1
DOCA	16	-36±2.5*	5.4±1.1	31±2.3

Values are mean ± SEM; n, number of cells
*P<0.01 when compared to control cells

Table 3-2. Mean Alterations in K^+ Current at 0 and +40 mV

		K_V		K_{Ca}	
Cell Type	*n*	0 mV	40 mV	0 mV	40 mV
WKY	7	2.5±0.03	11.0±0.3	1.9±0.1	14.2±1.5
SHR	7	0.1±0.01*	3.2±0.1*	2.8±0.2*	35.0±1.8*
SD	7	2.9±0.09	12.0±0.5	2.3±0.1	12.6±0.8
DOCA	7	0.2±0.03*	2.7±0.4*	3.7±0.4*	42.0±1.0*

Values are mean (pA/pF)±SEM; n, number of cells

*P<0.01 when compared to control cells

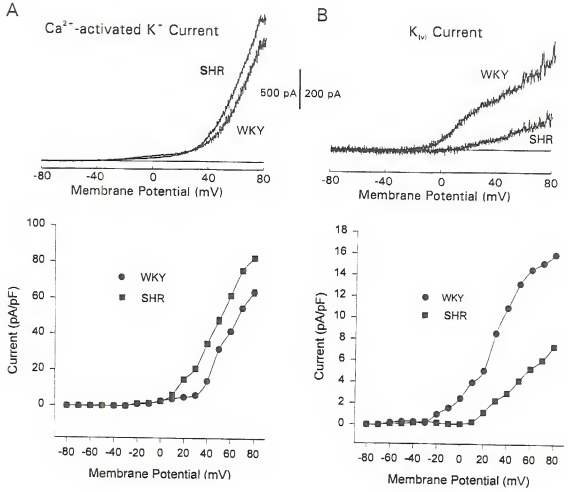

Figure 3-1. Isolation of Kv and K_{Ca} in WKY and SHR interlobar artery cells. (A) In the presence of 4-AP (10 mmol/L) and niflumic acid (100 μmol/L), SHR K_{Ca} was significantly greater than WKY. Similar results were obtained in seven cells. Voltage ramp and step depolarizations are shown in the upper and lower panels, respectively. (B) In the presence of charybdotoxin (100 nmol/L) and niflumic acid (100 μmol/L), WKY Kv was significantly greater than SHR Kv. Similar results were obtained in seven cells. Voltage ramp and step depolarizations are shown in the upper and lower panels, respectively. These two cells had similar membrane capacitances (WKY, 26 pF and SHR, 27 pF).

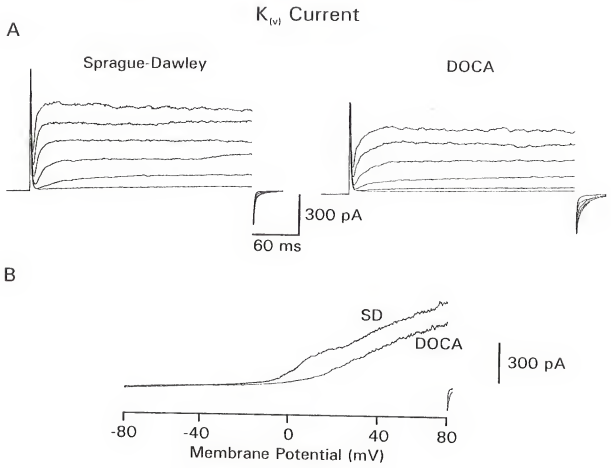

Figure 3-2. Isolation of Kv in SD and DOCA hypertensive interlobar artery cells. In the presence of charybdotoxin (100 nmol/L) and niflumic acid (100 μmol/L), SD Kv was significantly greater than DOCA hypertensive Kv during (A) step depolarizations (holding potential, -80 mV; test potential,-20 mV to 80 mV; 20 mV steps) and (B) ramp depolarizations (-80 to 80 mV, 4 seconds). The membrane capacitances of the two cells were similar (SD, 30 pF; DOCA 29 pF). Similar results were obtained in 7 cells.

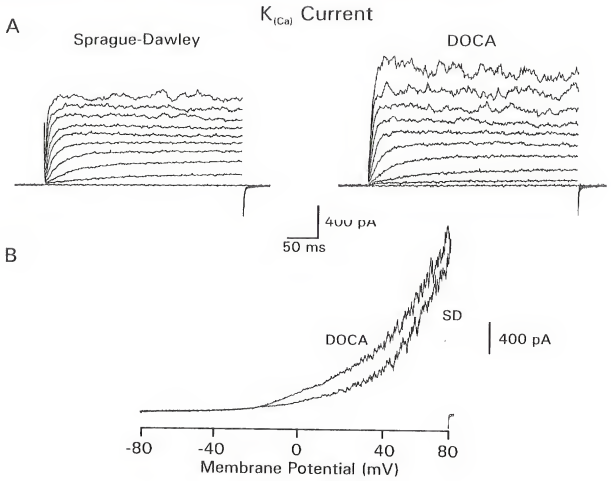

Figure 3-3. Isolation of K_{Ca} in SD and DOCA hypertensive interlobar artery cells. In the presence of 4-AP (10 mmol/L) and niflumic acid (100 μmol/L), DOCA hypertensive K_{Ca} was significantly greater than SD during (A) step (holding potential, -80 mV; test potential, -20 to 70 mV; 10 mV steps) and (B) ramp depolarizations (-80 to 80 mV, 4 seconds). These two cells had similar membrane capacitances (SD: 29 pF; DOCA: 30 pF). Similar results were obtained in seven cells.

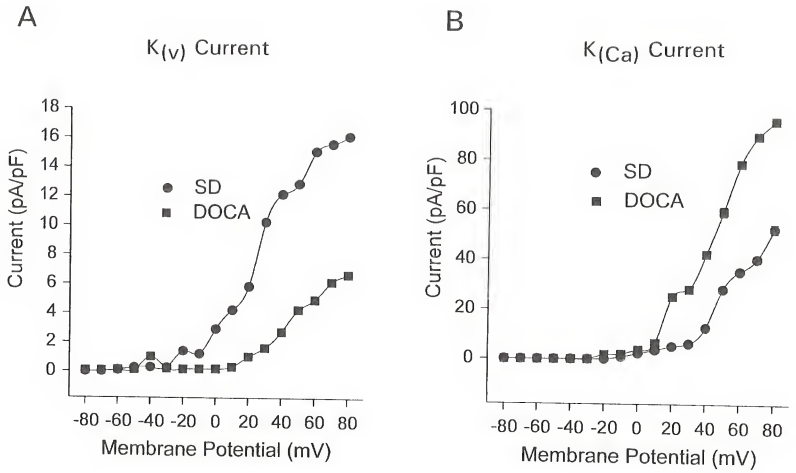

Figure 3-4. Mean current voltage relationships for Kv and K_{Ca} in SD and DOCA hypertensive cells. DOCA hypertensive Kv (A) and K_{Ca} (B) are significantly altered when compared to control (n=7). Error bars are the same size of the symbol and therefore are not visible.

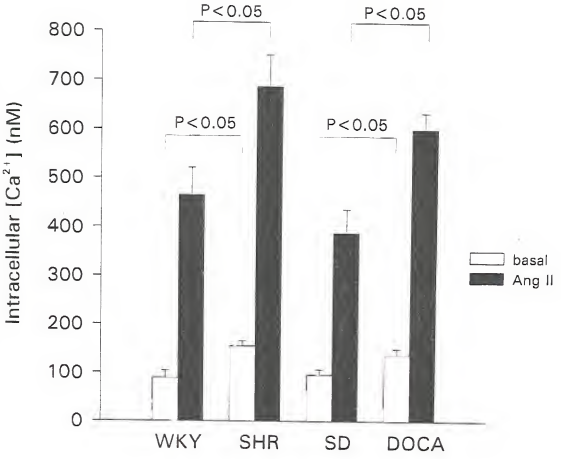

Figure 3-5. Basal and Ang II-simulated $[Ca^{2+}]_i$. Both basal and Ang II-stimulated $[Ca^{2+}]_i$ are significantly different from control (n=7, *p<0.05).

CHAPTER 4
PREVENTION OF RENOVASCULAR AND CARDIAC PATHOPHYSIOLOGICAL CHANGES IN HYPERTENSION BY AT_1 RECEPTOR ANTISENSE GENE THERAPY

Introduction

The elevation of systemic blood pressure (BP) associated with hypertension is a risk factor for cardiovascular disease and renal failure. Often it is the pathophysiological alterations and impairments associated with hypertension that lessen life expectancy. Pharmacological intervention has been relatively successful in normalizing the elevation in BP. However, the assumption that reduction of BP will totally reverse hypertension-induced pathophysiological changes remains unclear (Ruilope *et al.*, 1990; de Celentano *et al.*, 1993; Vogt *et al.*, 1993; Ruilope *et al.*, 1994).

The duration of treatment, age at which the antihypertensive therapy is initiated, site of administration, and specific agent used all appear to influence the reversal of the pathophysiological alterations associated with the disease (Linz *et al.*, 1989; Tschudi *et al.*, 1994; Schmieder *et al.*, 1996). In some instances, reversal of pathophysiological alterations may even be unfavorable such as when regression of left ventricular hypertrophy (LVH) and peripheral resistance occur in a disproportionate manner (Simko, 1994). Similarly, other reports have indicated that traditional antihypertensive agents can contribute to target organ injury by altering metabolic processes (i.e. hypercholesterolemia, glucose intolerance, hyperkalemia).

A role for the renin-angiotensin system (RAS) in the development of hypertension is well established in both humans and animal models such as the spontaneously hypertensive rat (SHR). Interruption of the RAS pathway, either by preventing the formation of Ang II (i.e. ACE inhibitor) or by blocking its actions at the level of the peptide receptor (i.e. AT_1 receptor antagonists), has been shown to reduce BP and protect against target-organ injury (MacGregor, 1992; van Zwieten, 1992; Kang *et al.*, 1994; Goldberg *et al.*, 1995; Berecek & Zhang, 1995). However, the attenuation or delay of non-hemodynamic pathophysiological impairments with these agents does not entirely reduce the risk to hypertensive patients (Vogt *et al.*, 1993; de Celentano *et al.*, 1993). In addition, chronic administration of traditional therapies is necessary for long term antihypertensive benefits. Required daily dosing and undesirable side effects such as sexual dysfunction, coughing, and lethargy diminish patient compliance.

We have previously established that retrovirally mediated delivery of AT_1-receptor antisense (AT_1R-AS) attenuates the development of high blood pressure on a long term basis (Iyer *et al.*, 1996; Lu *et al.*, 1997). A single injection of AT_1R-AS into neonatal SHRs prevented the development of hypertension up to 90 days after injection. Our objective in this study was to determine if this attenuation of high BP was associated with the prevention of renal and cardiovascular pathophysiological changes induced by the hypertensive state.

Materials and Methods

Preparation of Viral Particles Containing AT_1R-AS

A retroviral vector, LNSV, was used to deliver $AT_{1B}R$-AS into the rats (Lu *et al.*, 1995; Lu & Raizada, 1995). The $AT_{1B}R$-AS was cloned in the LNSV vector. It was

transfected to packaging cell line PA317 (American Type Culture Collection). After selection by G418, the medium containing viral particles that expressed AT_{1B}R-AS (LNSV-AT_1R-AS) was collected and used for all animal experiments. Viral particles that did not contain AT_{1B}R-AS (LNSV) were also prepared by the above protocol and used as a control.

Animals and Experimental Protocols

Five-day-old Wistar Kyoto (WKY) and SHRs were used in this study (Harlan Sprague Dawley). They were divided into three groups: vehicle (control), virus alone (LNSV), or virus containing AT_1R-AS (LNSV-AT_1R-AS). Treatments were injected via the cardiac route under methoxyflurane (metofane, Pitmin-Moore, Mundelein, IL) anesthesia. A bolus of 5×10^{10} plaque-forming units of viral particles in 10 µl of physiological saline was used per animal. There was an ~95% survival rate 24-48 hr after viral administration.

At the end of 120 days, rats from each group were used for determining the mean BP through an indwelling catheter implanted in the carotid artery essentially as described (Iyer *et al.*, 1996). Rats were anesthetized with a mixture of ketamine (30 mg/kg), xylazine (6 mg/kg), and acepromazine (1 mg/kg) administered intramuscularly (0.7 ml/kg). Direct blood pressure was recorded from the carotid artery catheter (PE-50) in free-moving, nonrestrained animals with a pressure transducer coupled to a Digi-Med blood pressure analyzer (Micro-Med, Louisville, KY).

Smooth Muscle Cell Isolation

Male rats were killed by decapitation and the right and left kidneys were removed and placed in cold, oxygenated (95% O_2-5% CO_2) physiological saline solution (PSS). Renal resistance vessels were identified as the fourth to fifth branch distal to the renal artery. These vessels were dissected free of fat and connective tissue under a dissecting microscope. The vascular fragments were then cut into small pieces (~ 4 mm) and incubated at room temperature in Ca^{2+}-free PSS for 30 minutes. The pieces were subsequently resuspended in Ca^{2+}-free PSS digestion buffer for 20-30 minutes at 37°C. The Ca^{2+}-free PSS digestion buffer contained (mg/15 ml) collagenase (151 units/mg Worthington Biochemical Corp., Freehold, NJ.) 4.5, bovine serum albumin 30, trypsin inhibitor 30, ATP (sodium salt) 1.7, and protease (type XXIV, Sigma Chemical Co., St. Louis, Mo.) 1.5. After the digestion, the pieces were removed from the digestion buffer, rinsed in Ca^{2+}-free PSS, and gently triturated until a large number of elongated smooth muscle cells were observed. The isolated cells were collected and stored at 4°C until used. Cells were used between 2 and 10 hrs. after isolation.

Current Recording and Analysis

Single cells were voltage-clamped, and membrane currents were measured using the whole-cell patch-clamp technique. Patch pipettes were made from borosilicate glass capillaries, pulled on a vertical puller (PP-83, Narishige Scientific Instrument Laboratory, Tokyo), and fire-polished with a microforge (model MF-83, Narishige); The pipettes had resistances of 3-5 MΩ when filled with the pipette solution.

Voltage-clamp command potentials were applied to the cells and membrane current recorded using an Axopatch-1D patch-clamp amplifier (Axon Instruments, Burlingame, CA). Membrane current was digitized on-line (10.0 kHz) with in analog-to-digital interface (Labmaster TL-1 DMA interface, Axon Instruments) and filtered at 2.0 kHz. Data analysis was performed with pCLAMP 6.0 software (Axon Instruments). All experiments were performed at room temperature.

Tension Measurements

A segment of the rat renal artery was dissected free and cleaned of fat and adventitia. Ring segments (3 mm long) were mounted onto two triangular tungsten wires (35 μm in diameter) and hung vertically in an isolated organ chamber (10 ml). The bottom triangle was mounted to a stable hook while the top triangle was attached to a Gould strain gauge. The bath was maintained at 37°C in PSS. A resting force of 1.5 g was applied to the vessels. Vessel segments were equilibrated for 1 hr with three exposures to phenylephrine (1 μmol/L). KCl, Phe, and ACh dose response curves were performed in a cumulative manner.

Assessment of Ventricular Hypertrophy and Cardiac and Perivascular Fibrosis

Animals were sacrificed, hearts excised, rinsed in 0.9% physiological saline, and total heart and ventricular weights were recorded. These weights were normalized to body weight (BW in g), and the ratios were compared among groups. In a separate series of experiments, the heart, liver, kidney, and adrenal gland were removed during deep anesthesia after arresting contraction by left ventricular injection of saturated KCl. These tissues were fixed in 3% freshly prepared paraformaldehyde in 0.15 mol/L phosphate

buffer and stored at 4°C for 24-72 hrs and then placed in phosphate buffer with 0.25 mol/L sucrose added (approximately 300 mOsM solution) at 4°C. Slices of right and left ventricles, liver, kidney and adrenal were dehydrated through a graded series of ethanol and xylene, and embedded in paraffin. Sections were cut at 5 μm thickness, stained with hemoatoxylin, eosin, and with aldehyde fuchsin Gomori trichrome and examined by light microscopy. Heart tissue was qualitatively evaluated for type (perivascular, focal or interstitial) and distribution (left or right ventricle, subendocardial, subepicardial) of connective tissue.

Solutions

The PSS used during cell isolation procedures and contraction experiments contained in mmol/L: NaCl 120, KCl 4.2, KH_2PO_4 1.2, $MgCl_2$ 0.5, $CaCl_2$ 1.8, glucose 5.5, and HEPES 30 (pH 7.35 with NaOH). For all whole-cell voltage-clamp experiments examining Kv currents, the bath solution contained in mmol/L: NaCl 125, KCl 4.2, KH_2PO_4 1.2, $MgCl_2$ 2.3, D-glucose 5.5, TEA 5.0, and HEPES 30 (pH 7.4 with NaOH). The pipette solution for the whole-cell experiments contained in mmol/L: KCl 140, $MgCl_2$ 0.5, EGTA 1.0, ATP (magnesium salt) 5.0, and HEPES 5.0 (pH 7.2 with KOH).

Statistics

Results are expressed as mean ± SEM. Statistical significance was evaluated using repeated measures ANOVA and Student's T-test for unpaired data. Differences were considered significant at $P<0.5$. Membrane currents were measured from the zero current level and normalized to cell capacitance.

Results

Effect of AT_1R-AS Treatment on Blood Pressure

A single intracardiac injection of LNSV-AT_1R-AS to neonatal rats prevented the increase in BP exclusively in SHRs at 120 days post-injection (fig. 4-1). The mean BP was 28.9% lower in SHRs treated with LNSV-AT_1R-AS than in untreated SH rats (116.3 ± 16.6 vs. 163.7 ± 6.0 mmHg; n=6, $P< 0.05$). In addition the mean BP measured in LNSV-AT_1R-AS treated SH rats was not significantly different from that of the normotensive WKY control rats (116.3 ± 16.6 vs. 115.6 ± 8.3 mmHg; n=6, $P>0.05$). This effect was specific for LNSV-AT_1R-AS treatment since treatment with LNSV alone had no significant effect on mean BP compared with control SHRs (n=6). Antisense treatment or vector alone did not affect the BP of WKY rats (n=6).

Effect of LNSV-AT_1R-AS Treatment on Renal Vascular Reactivity

Alterations in vascular contractile response are known to exist in the SHRs (Triggle & Laher, 1985; Bohr & Webb, 1988). LNSV-AT_1R-AS treatment prevented the alterations in renal artery reactivity measured in the SHRs. Enhanced contractile responses to both potassium chloride (KCl, Fig. 4-2) and phenylephrine (Phe, Fig. 4-3) were observed in the SHR model. A leftward shift in the KCl and Phe concentration-response relationships were observed in SHRs (Figs. 4-5A and B) when compared to WKY controls. The medium effective concentrations (EC_{50}) for KCl and Phe were 31.0% and 46.8% lower (n=6) in the SHR (24 mmol/L, KCl and 202 nmol/L, Phe) than in WKY(35 mmol/L, KCl, and 380 nmol/L, Phe) rats, respectively. The shifts in the concentration-response relationships for KCl or Phe were not observed in LNSV-AT_1R-

AS treated SHRs (EC_{50}: 34 mmol/L, KCl and 470 nmol/L, Phe) and LNSV alone (EC_{50}: 26 mmol/L, KCl and 132 nmol/L, Phe) did not change the renal artery contractile properties to KCl or Phe. Finally, an impaired endothelial-dependent relaxation of pre-contracted renal arteries was also observed in the SHR (Fig. 4-4). When the arteries were pre-contracted with Phe (500 nmol/L), the ACh dose response curve was right shifted and the maximal relaxation was decreased 36% (n=6) in the renal artery from the SHR (Fig.4-5C). The EC_{50} value for ACh-induced relaxation in SHR (143 nmol/L) renal artery was increased 4.7 fold compared to WKY (30 nmol/L). This shift was not seen in the LNSV-AT_1R-AS treated animals (73 nmol/L). However it was still present in SHR treated with LNSV alone (196 nmol/L).

Effect of LNSV-AT_1R-AS on Kv Current Density

We have previously shown that Kv current density was decreased in vascular smooth muscle cells from the interlobar arteries of the SHR when compared to WKY control (see chapter 3). Figure 4-6 illustrates the characteristics of Kv current in single cells isolated from renal resistance vessels of WKY and SHRs. During voltage step depolarizations in the presence of 10 mmol/L TEA (to block the K_{Ca}), Kv current displayed two components. The first is a rapidly activating and inactivating component which we termed the peak component of Kv current. The second is a slow inactivating sustained component.

Under the above conditions, the Kv current was reduced in cells isolated from the SHR. This decrease was not evident in SHRs treated with LNSV-AT_1R-AS but was apparent in those treated with LNSV alone. Figure 4-7 shows the mean current voltage relationship for both the peak (Fig. 4-7A) and sustained (Fig. 4-7B) component of Kv

current. In each experiment the Kv current, normalized to cell capacitance, was significantly less in the cells from SHRs as compared to those obtained from WKY controls. Again, this decrease was not observed in cells from LNSV-AT_1R-AS treated SHRs. It should be noted that the cell capacitance was not significantly different between all of the groups tested.

Effect of LNSV-AT_1R-AS on Heart Weights and Cardiac Morphology

Ventricular weights were recorded for all groups and normalized to body weight. The ventricular weights of SHRs treated with LNSV alone were significantly greater than the weights recorded for the normotensive WKY (Table 4-1). This increase was not seen in SHRs treated with LNSV-AT_1R-AS. There was no difference in the weights of the LNSV-treated and LNSV-AT_1R-AS-treated WKY ventricles. Figure 4-8 shows the effect of LNSV-AT_1R-AS on cardiac and perivascular fibrosis. Panel A shows photomicrographs of representative sections taken from left ventricular subendomyocardiurn from (top) WKY, SHR and LNSV-AT_1R-AS-treated SHRs , respectively. Multiple focal areas of fibrosis were evident in hearts from SHRs (indicated by arrows). This phenomenon was not seen in hearts from the WKY or LNSV-AT_1R-AS-treated SHRs. Panel 4-8B depicts series of photomicrographs of sections from mid-myocardium showing arterioles of less than 100 μm in diameter. Intramyocardial small coronary arteries and arterioles from SHRs displayed dense perivascular fibrosis. This was not seen in coronary vessels from WKY or LNSV-AT_1R-AS-treated SHRs. No lesions were present in liver, kidney, or adrenal tissues from any animal.

Discussion

The results of this study demonstrate that delivery of AT_1R-AS prevents the development of hemodynamic and pathophysiological alterations associated with hypertension in the SHR. Neonatal SHRs which were given a single intracardiac injection of AT_1R-AS did not develop an elevation in mean BP, altered renal vascular reactivity, decreased smooth muscle Kv channel current density, left ventricular hypertrophy, or cardiac and perivascular fibrosis. These novel data suggest that interruption of AT_1 receptor in the developing SHR, using a virally-mediated gene delivery system, may be used to prevent hypertension and its associated renal and cardiovascular risk factors.

The role of the kidney in the control of fluid volume and BP implies its participation in the development of essential hypertension. The results of earlier renal transplant studies in humans and animals suggest that in a proportion of hypertension cases there exists structural or functional alterations in the kidney (Dahl *et al.*, 1972; Kawabe *et al.*, 1978; Curtis *et al.*, 1983; Rettig *et al.*, 1990). In addition, evidence indicates that regulation of renal blood flow is an important element of the hypertensive process. Multiple studies have demonstrated that in hypertension there exists an enhancement in renal vascular tone (Guyton *et al.*, 1990; Ruilope *et al.*, 1994). This elevation in tone may lead to increased renal vascular resistance, a common observation in hypertension, and an important factor in shifting pressure natriuresis and elevating BP. Possible mechanisms responsible for the increase in renal vascular resistance include: (1) an enhanced contractile sensitivity to vasoactive agonists; (2) an impaired endothelial dependent relaxation; (3) an increased Ca^{2+} transport across vascular smooth muscle

membranes; (4) an altered ion channel activity in vascular smooth muscle; and (5) smooth muscle hypertrophy or hyperplasia (Khalil *et al.*, 1989). Our results show that both KCl and Phe produced an enhanced contractile response in renal arteries of SHRs. However, this was not observed in SHRs treated with AT_1R-AS. The augmented contractile response to vasoactive agents appears to depend on the vascular bed tested and the type of vasoconstrictor agent (Berecek *et al.*, 1980; Silva *et al.*, 1996). However, this does not diminish the significance of the result that alterations in both electro-mechanical coupling, which is membrane potential-dependent (KCl), and pharmaco-mechanical coupling, which is agonist-dependent (Phe), were prevented with AT_1R-AS treatment.

A second mechanism which may contribute to increased renal vascular resistance and/or decreased renal blood flow is endothelial dysfunction. It is well established that endothelial-derived vasoactive agents regulate blood flow through vessels by controlling the contractile state of vascular smooth muscle (VSM) cells (Vanhoutte *et al.*, 1986). One such substance, nitric oxide (NO), is released by the endothelium to cause relaxation of the VSM cells (Furchgott & Zawadzki, 1980). It has recently been reported that ACh induced relaxation of coronary arteries is mediated by endothelial-derived NO (Simko, 1994). Our results show that there exists an impaired endothelial dependent relaxation to ACh in the renal artery of SHRs. This alteration is prevented in SH rats treated with AT_1R-AS. Reports of altered endothelial function appear to depend on a number of factors including vascular bed, model of hypertension, and constrictor agent used (Luscher *et al.*, 1988; Li & Bukoski, 1993; Fuchs *et al.*, 1996). Some reports suggest that the altered endothelial relaxation in hypertension is not due to impaired NO release but rather to changes in release of vasoconstricting prostaglandins (Luscher *et al.*, 1988; Li

& Bukoski, 1993). Regardless of the mechanism, our data demonstrate an impaired endothelial relaxation in the SH rats which was prevented with AT_1R-AS treatment.

A third mechanism by which renal vascular resistance may be increased in hypertension is altered ion channel function in VSM cells. It has been demonstrated that Kv current modulates membrane potential (Fleischmann *et al.*, 1993; Knot & Nelson, 1995). Tonic changes in membrane potential regulate Ca^{2+} influx and hence contractile tone in VSM cells. Blockade or reduction of this current may lead to increases in tone and ultimately to an increase in vascular resistance. There is substantial evidence for electrophysiological changes in hypertension (Hermsmeyer, 1976; Rusch & Hermsmeyer, 1988; Rusch *et al.*, 1992; England *et al.*, 1993; Ohya *et al.*, 1993; Wilde *et al.*, 1994). We have previously demonstrated that the Kv current is decreased in rat renal resistance vessels from both a genetic and nongenetic model of hypertension (see chapter 3). Herein we show that this alteration can be prevented in the SHR by treatment with AT_1R-AS. The parallel between the prevention of the Kv current alterations and normalization of blood pressure suggests that, in hypertension, the alterations in VSM cell K^+ channel function represents feedback mechanisms occurring secondary to the change in arterial blood pressure (Rusch & Runnells, 1994) or distal to a RAS triggered event.

Cardiovascular ultrastructural changes are a major risk factor for morbidity and mortality in hypertension. Ventricular hypertrophy is a compensatory response to the increased pressure load attributable to the elevation in peripheral resistance (Vogt *et al.*, 1993). While traditional pharmacological therapies have been shown to be effective in controlling and at times reversing these pathophysiological changes, there are often times when this approach is not successful (de Celentano *et al.*, 1993; Vogt *et al.*, 1993). It is

important to note that regression of LVH has been reported in only 50% of patients treated with traditional antihypertensive drugs and that it is difficult to determine which patients will benefit from these therapies (de Celentano *et al.*, 1993). Combination therapies may improve benefits due to the additive BP lowering effects of some antihypertensive agents. However, this may also increase the number and intensity of undesirable side effects and hence further decrease patient compliance. We found an increase in ventricular mass in SHRs treated with LNSV vector alone. However, this ventricular hypertrophy was prevented with AT_1R-AS treatment. Other structural alterations (i.e. cardiac and perivascular fibrosis) were also prevented with AT_1R-AS treatment.

Finally, evidence suggests that simply normalizing the BP by pharmacological means is not sufficient to completely regress pathophysiological changes associated with the hypertension. AT_1R-AS gene therapy may be superior. It results in the prevention of the increase in mean BP and the associated pathophysiological impairments in hypertension. It may also offer an alternative to the compliance problem and complications of vascular and target-organ injury. Finally, the AT_1R-AS therapy does not produce a significant increase in plasma Ang II levels compared with the antihypertensive effects of losartan, an AT_1 receptor antagonist (Lu & Raizada, 1995). Therefore AT_1R-AS therapy may have prolonged antihypertensive effects without the possible adverse side effects of elevated plasma Ang II.

A role for RAS in the development or maintenance of hypertension is well established (Berecek *et al.*, 1987; Phillips, 1987; MacGregor, 1992; Wu & Berecek, 1993; Kang *et al.*, 1994). Our results confirm a fundamental role for AT_1 receptors in

both the development of high BP and the production of pathological organ damage. The interruption of the RAS pathway at an early age using AT_1R-AS offers the potential to prevent the development of hypertension and its associated pathophysiological alterations with a single antisense treatment. Of course this approach depends on the identification of genetic determinants of hypertension or upon the demonstration of reliable pre-hypertensive risk factors. Studies are now underway to determine if this gene delivery approach can be used to reverse the hemodynamic and pathophysiological alterations in the adult hypertensive animal.

TABEL 4-1. Ventricular weights normalized to body weights in WKY and SHR

Group	n	VW (g)	VW/BW (mg/g)
WKY + AT_1R-AS	4	1.05 ± 0.11	3.2 ± 0.09
WKY + LNSV	3	1.07 ± 0.20	3.0 ± 0.32
SHR + AT_1R-AS	6	1.14 ± 0.08	3.6 ± 0.13
SHR+LNSV	3	1.54 ± 0.15 *	4.8 ± 0.85 *

Values are means ± SE: VW=ventricular weight, BW= body weight, *P<0.05

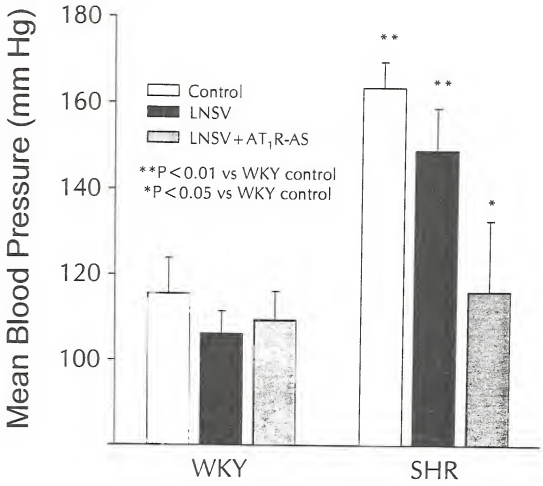

Figure 4-1. Effect of LNSV-AT_1R-AS treatment on mean blood pressure in WK and SH rats. Five-day-old WKY and SH rats treated with physiological saline (control), LNSV, LNSV+AT_1R-AS were allowed to mature for 120 days. Mean blood pressure was measured though catheters implanted in the carotid artery. A single injection of LNSV+AT_1R-AS into neonatal SH rats prevented the increase in BP. There was not a significant effect of LNSV or LNSV+AT_1R- AS on the mean BP in the WKY. Data are expressed as mean ± SE (n=6).

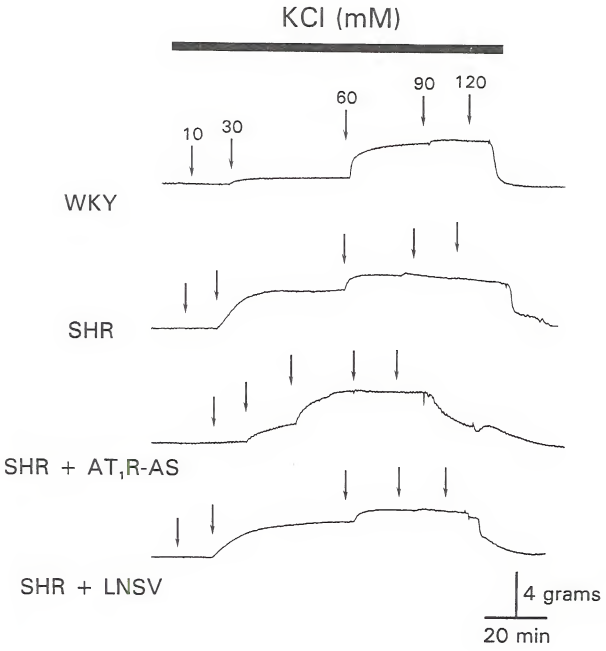

Figure 4-2. Prevention of the alteration in the KCL concentration-response relationship of rat renal artery in hypertension with AT_1R-AS. Shown are representative examples of contractions in the rat renal artery from WKY, SHR, SHR + AT_1R-AS, and SHR + LNSV. Arrows indicate time of intervention.

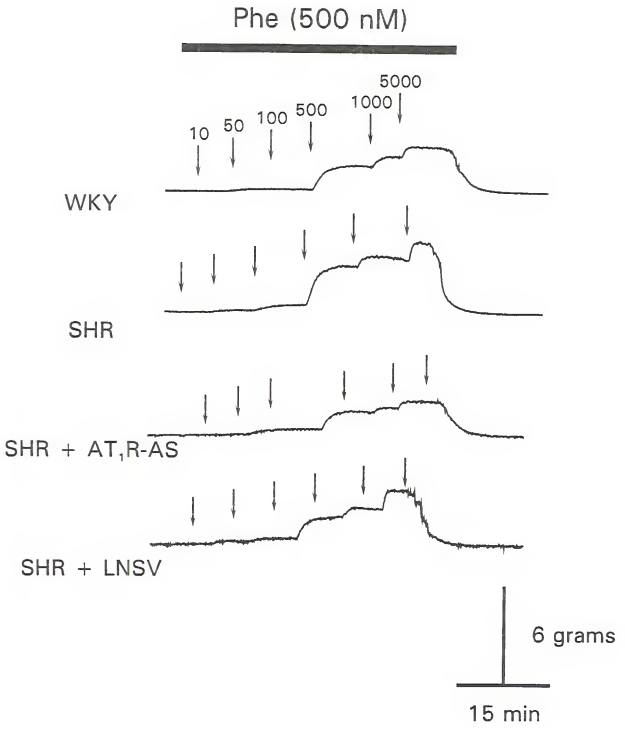

Figure 4-3. Prevention of the alteration in the Phe concentration-response relationship of rat renal artery in hypertension with AT_1R-AS. Shown are representative examples of contractions in the rat renal artery from WKY, SHR, SHR + AT_1R-AS, and SHR + LNSV. Arrows indicate time of intervention.

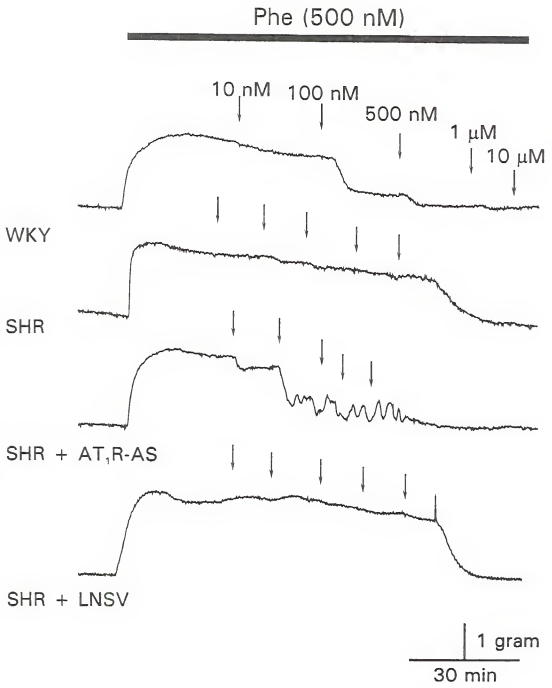

Figure 4-4. Prevention of the alteration in the acetylcholine concentration-response relationship of rat renal artery in hypertension with AT_1R-AS. Shown are representative examples of contractions in the rat renal artery from WKY, SHR, SHR + AT_1R-AS, and SHR + LNSV. Vessels were pre-contracted with Phe. Arrows indicate time of Ach intervention

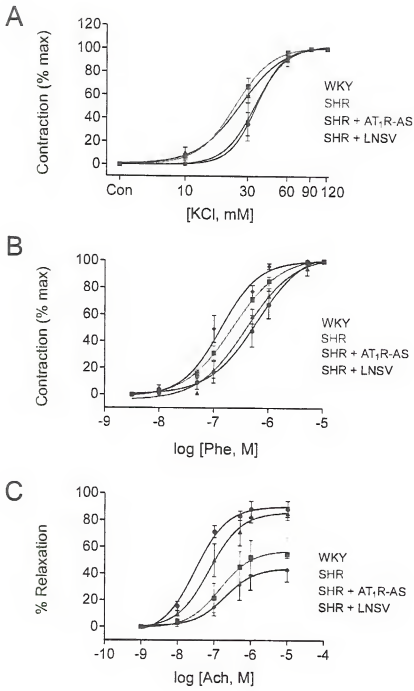

Figure 4-5. Mean concentration-response relationship to KCl (A), Phe (B) and ACh (C) in rat renal artery. The Phe and KCl dose response curve was shifted to the left in SHR (■) and SHR + LNSV (◆) when compared to WKY (●) and SHR + AT_1R-AS (▲). The ACh dose response curve was shifted to the right and the maximal relaxation was decreased in the SHR and SHR + LNSV when compared to WKY and SHR + AT_1R-AS. This shift was prevented in all intervention with delivery of AT_1R-AS. Data are presented as mean ± SE (n=6).

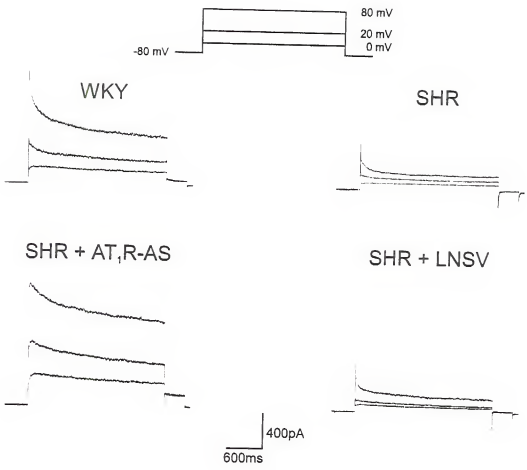

Figure 4-6. Characteristics of Kv current in single vascular smooth muscle cells isolated from rat renal resistance arteries. The Kv current was reduced in cells from SHR and SHR + LNSV-treated rats. Delivery of AT_1R-AS prevented this decrease. The voltage protocol is displayed at the top. The membrane capacitances of all 4 cells were similar (WKY, 28 pF; SHR, 28 pF; SHR+AT_1R-AS, 29 pF; SHR+LNSV, 28 pF).

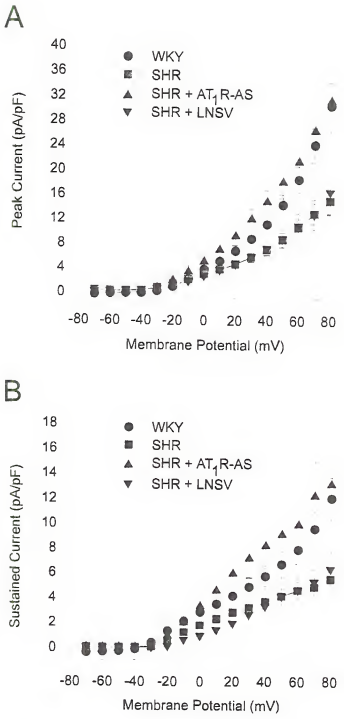

Figure 4-7. Mean current-voltage relationships for peak (A) and sustained (B) components of Kv current. There was no significant difference in current density in the WKY (●, n=7) and SHR+AT,R-AS (▲, n=6) treated rats. However, the Kv current density for both the peak and sustained components was significantly lower in the SHR (■, n=12) and SHR+LNSV treated (▼, n=6) animals.

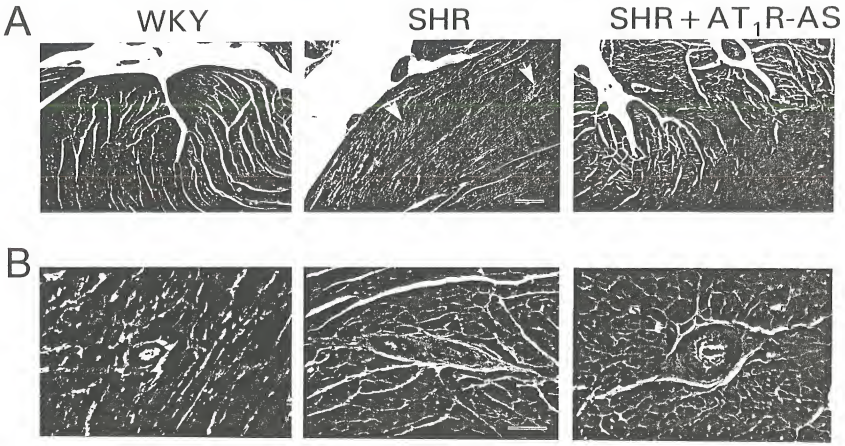

Figure 4-8. Effect of AT_1R-AS on cardiac and perivascular fibrosis. (A) Photomicrographs of sections taken from left ventricular subendomyocardium from WKY, SHR, and SHR+AT_1R-AS, respectively. Photographs were 50X magnification at 4X obj. Calibration bar 12 mm = 250 μm. Arrows in SHR panel indicate multiple focal areas of fibrosis. This phenomenon was not seen in hearts from WKY or SHR+AT_1R-AS. (B) Sections from mid myocardium showing small arteries and arterioles (<100 μm diameter) from WKY, SHR and SHR+AT_1R-AS treated hearts. Photographs were taken at X308 magnification at X40 objective. Calibration bar 15 mm = 50 μm. Vessels from SHR demonstrated dense perivascular fibrosis. This was not seen in vessels from WKY or AT_1R-AS treated rats.

CONCLUSIONS

Results of this study characterize the Kv current in rat renal resistance arterioles and provide the first *in vivo* evidence for heteromeric formation of K^+ channels in vascular smooth muscle. In addition, this study details changes in Kv current and membrane potential in genetic and nongenetic models of hypertension. Finally, this work demonstrates that the decrease in Kv current density as well as other renovascular and cardiac pathophysiological alteration in hypertension, can be prevented using AT_1 receptor antisense gene therapy.

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BIOGRAPHICAL SKETCH

Jeffrey R. Martens was born on December 16, 1968, in Plymouth, Michigan, to Randall H. Martens and Sharon L. Martens. He received his elementary education in Henry Ruff Elementary School, Garden City, Michigan. In 1987, he graduated salutatorian of his high school class, Hudson Senior High School, Bayonet Point, Florida. He received a B.S. degree in chemistry from the University of Florida, 1993. In 1994, he began his doctoral studies in pharmacology at the University of Florida, under the mentorship of Dr. Craig H. Gelband. In the August of 1997, he married Lori A. Bliss from Ann Arbor, Michigan. After completion of his dissertation, he will begin postdoctoral work with Dr. Michael M. Tamkun at Colorado State University in Fort Collins, Colorado.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Craig H. Gelband, Chair
Assistant Professor of Physiology and
Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Luiz Belardinelli
Eminent Scholar of Pharmacology
and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jeffrey Harrison
Assistant Professor of Pharmacology
and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Philip Posner
Professor of Physiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Charles Wingo
Professor of Medicine

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1998

Dean, College of Medicine

Dean, Graduate School